CONNECTIVE TISSUES

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Third Conference on Connective Tissues

JOSIAH MACY, JR. FOUNDATION CONFERENCE PROGRAM

As an introduction to these Transactions of the Third Conference on Connective Tissues, I should like to outline what it is that the Foundation hopes to accomplish by its Conference Program We are interested, first of all, in furthering knowledge about connective tissues and to this end the participants were brought together to exchange ideas, experiences, data, and methods. In addition to this particular goal, however, there is a further, and perhaps more fundamental, aim which is shared by all our conference groups This is the promotion of meaningful communication between scientific disciplines

The problem of communication between disciplines we feel to be a very real and a very urgent one, the most effective advancement of the whole of science being to a large extent dependent upon it Because of the accelerating rate at which new knowledge is accumulating and because discoveries in one field so often result from information gained in quite another, channels must be established for the most relevant dissemination of this knowledge

The increasing realization that nature itself recognizes no boundaries makes it evident also that the continued isolation of the several branches of science is a serious obstacle to scientific progress Particularly is it so in medicine that the limited view through the lens of one discipline is no longer enough. For example, today medicine must be well versed in nuclear physics because of the tracer techniques and the injury which can result from radiation At the other extreme, medicine is certainly a social science and, through mental health, must be concerned with economic and social questions. The answer, then, is not further fragmentation into increasingly isolated specialties, disciplines, and departments, but the integration of science and scientific knowledge for the enrichment of all branches This integration, we feel, can be encouraged by providing opportunities for a multiprofessional approach to given topics.

Although the fertility of the multidiscipline approach is recognized, adequate provision is not made for it by our universities, scientific societies, and journals And perhaps the presence of other hindering factors must be admitted Partly semantic in nature, they may also to some degree be psychological. Admittedly, it is oftentimes difficult to accept data derived from methods with which one is unfamiliar By

making free and informal discussion the central core of our meetings, we hope to achieve an atmosphere which minimizes as much as possible these emotional barriers.

Thus, our meetings are in contrast to the usual scientific gatherings. They are not designed to present neat solutions to tidy problems but to elicit provocative discussion of the difficulties which are being encountered in research and practice. For this reason, we ask that the presentations be relatively brief and that emphasis be placed on discussion as the heart of the meeting. Our hope is that the participants will come prepared not to defend a single point of view but to take advantage of the meeting as an opportunity to speak with representatives of other disciplines in much the same way that they would talk with their own colleagues in their own laboratories.

We have, now, thirteen groups functioning under the Conference Program, on the following topics adrenal cortex, aging, blood clotting, cold injury, connective tissues, consciousness, cybernetics, infancy and childhood, liver injury, metabolic interrelations, nerve impulse, renal function, and shock and circulatory homeostasis. When a new conference is organized, the Chairman, in consultation with the Foundation, selects fifteen scientists to be the nucleus of the group, and every effort is made to include representatives from all pertinent disciplines From time to time new members are added by the group to fill gaps in viewpoint or technique A limited number of guests are invited to attend each meeting, but, for the purpose of promoting full participation by all members and guests, attendance at any meeting is limited to twenty-five. It is inevitable that in no topic can we possibly include more than a small fraction of the key investigators in the field, and one of the difficulties in forming a group like this is that it is necessary to leave out so many people whom we would like to include

The transactions of these meetings are recorded and published. This is done because the Foundation wishes to make current thinking in a field available to all those working in it, and because it believes that conveying to those in other fields who are concerned with science, for example, government officials, administrators, etc., the essential nature of scientific research is also an important problem in communication Logic is a vital aspect of science, but equally essential is the intuitive or creative aspect Research is as creative as the painting of a portrait or the composing of a symphony. Although logic is, of course, necessary in order to rearrange, to test, and to validate, research thrives on creativity which has its source in unconscious, nonrational processes. Unfortunately, however, in the finished products which are presented

to the world through research reports this integral part of scientific endeavor is shriveled by the cold, white light of logic By preserving the informality of our conferences in the published transactions, we hope to give a truer picture of what actually goes on in the minds of scientists and of the role which creativity plays

FRANK FREMONT-SMITH, M D.
Medical Director

CONNECTIVE TISSUE STAINING

R. D. LILLIE

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DR FOOT DID ME THE HONOR a year or so ago of calling me the "technician of pathology." If I have attempted to assume the mantle of Karl Weigert and Frank B Mallory, I hope I have done it with enough attention to fact and not too much to theory, because fact is eternal and theory frequently may change from one moment to the next.

György Are you sure that facts are eternal?

Bauer: What are facts?

Lillie: Recently, I read with great pleasure an article written by Rudolf Virchow (1) in 1846 His description of what he did and what happened is still good, but the latter part of his article in which he speculated on what the facts meant is not understandable today. It is simply in that sense that I say that accurate observation and description is of lasting worth After Karl Weigert had explained one of his new methods, a student came to the podium and said, "But, Professor, what is the theory of this method?" Dr Weigert is reported to have said, with due solemnity, "Ach, elie Theorie! You should ask my nephew, Paul, about that!"

György Well, his nephew did fairly well I don't know anyone who was better with theory than Paul Ehrlich

Lillie Oh, yes. But I could remark that in modern histologic technique many of the methods of Weigert are still in use, while there is very little mention of the theories of Ehrlich

Fremont-Smith For every fact, there are certain basic assumptions which, in turn, are based on theory Therefore, all facts are relative in that sense and are open to modification; they have limitations.

Lillie. Yes But I do intend, in discussing methods for staining connective tissue, to present the facts and the results attained with them, and, after that, we can speculate on what they mean It is entirely legitimate to speculate after the observations, the methods of observation, and the limitations of those observations have been stated

Connective tissue I shall consider as a complex of various kinds of fibrous structures, basement membrane, and the so-called ground substances, going first into the general group of collagen, reticulum, and

basement membrane. I group them together since many of the methods used for their demonstration demonstrate all three. Collagen was initially identified as fibrillar material which swelled in acetic acid and hydrolyzed to form gelatin, a definition which is still good There has been a multiplicity of methods devised for the differential staining of collagen, varying greatly in their specificity and in their completeness of staining, as well as in their apparent basic principles I say "apparent" for reasons that I shall make plain later. Seemingly, the oldest differential connective tissue stain is picroindigocarmine. It was introduced by Jullien (2) in 1872, forgotten, and reintroduced by Ramón y Cajal (3, 4) in 1896, who generally receives credit for the method (5, 6, 7) Jullien made a saturated solution in water of indigocarmine, which is a sulfonated indigo acid dye, acidified with oxalic acid and mixed with concentrated picric acid solution The solution gave bluish connective tissue and greenish-yellow muscle and cytoplasm. The acid was important. A number of workers have used the same method since Nigrosin was substituted by Martinotti (8) in 1895 for the indigocarmine, and it gave similar color contrasts although the blue was slightly different in shade.

Freeborn (9) used the same method a little later. Van Gieson (10), in 1889, reported his use of acid fuchsin, picric acid mixtures He put a few drops of saturated acid fuchsin solution into saturated aqueous picric acid to produce a dark garnet color. His successors (11, 12, 13, 14, 15, 16) very soon specified how much acid fuchsin and how much picric acid to put in, and the usual proportions have varied from 5 parts of 1 per cent acid fuchsin to 10 parts in 100 of the mixture which was saturated, or nearly saturated, with picric acid (17). It seems best to specify those quantities in terms of milligrams of acid fuchsin per 100 ml. of mixture, and the proportion that we use is 1 of 1 per cent, as a rule, or 100 mg per 100 ml, which gives a deeper red to the connective tissue than the 05 of 1 per cent often employed, although it seems a little less specific If some hydrochloric acid is put into such a mixture, it precipitates some picric acid, which is not important, and the method becomes somewhat more specific. If more acid fuchsin is added, along with the hydrochloric acid, it becomes even more selective and will stain almost all of the connective tissue. The same thing is true of methyl blue and anilin blue mixtures with the picric acid These are also very successful, and they are probably the most selective of the simple methods for demonstrating connective tissue.

These observations of the influence of H ion, apart from the picric acid itself, are substantiated by Curtis (18) who substituted a number of yellow nitro compounds for picric acid and found that trinitro compounds of other sorts had about the same color effects and about the

same selectivity as those obtained with picric acid As to the dinitro compounds, which were less acid, there were not pH measurements in 1903 so it cannot be said what these would have been, however, the dinitro compounds were much less successful in producing differentiation

If the pH level of picric acid, acid fuchsin mixtures is raised from the usual 1 9 by the addition of salts of one sort or another, the mixtures become progressively less selective for connective tissue Very poor differentiation is obtained if ammonium picrate is used in place of picric acid Again, the importance of acidity must be stressed

Curtis theorized to a considerable extent on the nature of the dye used in picric mixtures He thought that when triphenylmethane dyes, which are typified in usual practice by acid fuchsin, anilin blue water-soluble, and methyl blue water-soluble, were sulfonated not on the triphenylmethane nucleus but on phenyl rings in side chains substituted into the amine groups, these were not successful We tried to duplicate this and were not convinced that his observations were accurate in that particular respect Anilin blue, which is sulfonated, according to our dye chemists, in side chains, is one of the best of the connective tissue stains for picric mixtures, as well as for the phosphotungstic or phosphomolybdic

Curtis obtained very good results with a number of azo dyes, and some of them in my hands have also been quite successful My disagreements with Curtis perhaps may be assigned to the fact that the manufacturers change the names of the dyes from time to time, and there are also differences in the purity of the dye samples available to each of us, that is, if I had a methyl blue which was 90 per cent active dye, and he had one that was 40 per cent active dye, which is entirely within the range of possibility, he might have thought it a very poor dye, and I might have thought it very good I do find that with different samples of methyl blue and of anilin blue, the amount that is put into picric mixtures must be varied in order to achieve successful differentiation It can run anywhere from 20 to 120 mg per 100 ml

Fremont-Smith Would you repeat your definition of collagen?

Lillie Collagen is a substance forming the fibers of the connective tissue which swells on treatment with a dilute acetic acid and which hydrolyzes to gelatin on boiling The definition is not mine

Fremont-Smith It would be interesting to know how many here would subscribe to that as being, from their point of view, a correct statement, and, secondly, whether we may not have to define collagen from several different points of view, not from one alone

Lillie Of course, there are more substances than collagen which swell in acetic acid and hydrolyze to gelatin Ossein, the framework

of bone, does so and it shares many of the histochemical reactions of collagen It also yields gelatin

Porter: It is collagen, isn't it?

Lillie: Yes

Gyorgy: How do you know that basement membrane is collagen?

Lillie: I don't and I didn't say so.

Gyorgy: Connective tissue contains many substances in addition to collagen.

Lillie: Oh, yes, I am sure it does I intend to discuss the multiplicity of the fibrous substances.

Bennett: Where do you plan to put ground substance with respect to collagen and basement membrane?

Lillie: *Ground substance is something about which I know comparatively little*

Fremont-Smith: Isn't it by definition that which we don't understand and hence call ground substance?

Lillie. Perhaps it could be called the amorphous or gelatinous or semifluid material which lies between the demonstrable fibrils and in which they may be imbedded By many of our methods, it is not evident; it cannot be seen It was neglected for a long time on that account

Bennett If that is the way you regard it, then where will you put basement membrane in this spectrum?

Lillie: As an organized structure, applied against endothelia and epithelia, between them and the fibrillar connective tissue. I think of it as a hyaline membrane, for reasons that I will develop later.

Bauer: And hyalin is what?

Lillie: When we first looked at some of the preparations which brought us to the view that basement membrane was distinct from the condensation of the connective tissue fibrils, we said they had the appearance of cellophane tubes. Does that convey the idea? One follows them in optical focus as apparently continuous membranes, flat sheets of material.

Bauer: We all wish we knew more about the land of the unknown These terms have come into use but we have only slight knowledge of composition and molecular structure, and any number of things, as regards some of these.

Lillie: We are acquiring more information as we go along about each

Robb-Smith: Is it not true that Dr Lillie's definition of collagen would be universally acceptable? Obviously, it is a limited one, but it is far safer to have it be so

Bauer: It is much more limited than the information at hand.

Robb-Smith Then can't you put another brick on, rather than make it too complicated at the beginning?

Bauer A brick can be added concerning molecular structure, another brick concerning amino acid composition, although the knowledge is necessarily incomplete.

Lillie: But amino acid varies to some extent with the source of collagen which is analyzed

Bauer: Right you are

Lillie So we cannot go too far into the details of amino acid constitution. We know that certain amino acids are prominent in the composition of all the things that we call collagen

Bauer And amino acid analyses are anything but recent

Meyer At previous meetings, and especially in the literature on x-ray diffraction and electron microscopy, the definition of collagen was quite different from the one Dr Lillie gives Under his definition, ichthyocol would not be classified as a collagen Maybe it ought to be stressed that his definition applies to mammalian collagen or collagen of certain species

Lillie X-ray diffraction definitions of collagen would be quite acceptable could it be certain that they comprised all the things which the morphologist has been calling collagen. The morphologist has probably been using the term as long as the biochemist

Bauer: But the morphologist has been using the term collagen without being sure what it is

Lillie: The morphologist has looked at certain wavy fibers in connective tissue and he knows that if he boils them in acidulated water, he gets gelatin out of them. The term was used before the amino acid analyses were done, I am sure, and I have not seen any good reason why it should be changed

Fremont-Smith: Your definition is a definition which, if I understand it correctly, involves two disciplines It involves microscopy and bio chemistry Therefore, it is a definition which is limited in frame of reference, unless it is necessary to say that physics is involved in the use of light Is that right?

Lillie I have no quarrel with allowing qualifications to come in from other disciplines

Fremont-Smith The point I wanted to make is that perhaps a definition of any substance should come from as many points of reference as possible It might be conceivable to have twenty-five definitions, each one of which would be a correct but limited definition and would have value for a particular purpose in terms of the goal for which it is being used

Lillie All the definitions of collagen rest primarily on the opinion

of the anatomist that this is connective tissue which he is giving to the chemist to analyze

Robb-Smith: Thus, it is absolutely vital that the actual material given the chemist or the electron microscopist can fulfill the criteria that Dr Lillie has put forward.

Lillie. The first criterion is the anatomical one in the selection of material to be examined by any method.

Fremont-Smith: If we were to qualify our use of the term each time by saying "collagen, anatomical," or "histochemical," or "electron microscope," this would immediately identify the frame of reference.

Mirsky. So long as the definition is operational, what difference does it make as to the particular frame of reference?

Fremont-Smith: I just want to have it stated that it is operational.

Wyckoff. I would be inclined to say that Dr. Lillie's is the parent definition and that other disciplines, in subsequent investigations of collagen, must ask to what degree their material corresponds to this definition and whether or not exceptions are found to it. For instance, from the standpoint of present-day morphologic methods, I think we would all say that fish collagen was collagen, in the sense that, using these methods, w [illegible]

Meyer [illegible]
does not s [illegible]
tion

Wyckoff There are forms of rat tendon which also go into complete solution, if they are selected properly.

Meyer. That depends on the concentration of acetic acid

Bauer: They all have the same molecular structure when examined under an electron miscroscope.

Porter: The same apparent structure, yes.

Lillie: The business of swelling and going into solution is often simply a question of time and temperature. A bone, if put into hydrochloric acid and a 56° incubator, decalcifies, swells, dissolves, and then disappears completely

[illegible]

process.

Meyer Of course.

Lillie. I would not say that going into solution was a fundamental difference [illegible] into solution, too, provided the [illegible] the proper temperature.

Ragan. [illegible]

Lillie: [illegible] latin or glue is produced

Bauer: If a little glycoprotein is added, will the fiber precipitate out of the solution?

Wyckoff: The material may or may not be collagen after it has gone into solution

Lillie Hydrolyzed far enough, it consists of amino acids

Bauer: According to one of your definitions, it would still be collagen, wouldn't it?

Wyckoff· It is collagen

Bauer: The fibers are formed again

Meyer· Would you agree, Dr Lillie, that collagens, to put it in the plural, are fibers which give gelatin?

Lillie I have no objection

We come now to a second large group of specific connective tissue stains, based on the Mallory (12, 13, 19, 20, 21) anilin blue method with phosphomolybdic and phosphotungstic acid Mallory first used the one acid, and then the other Various people have used either, or mixtures of both Because the dye chemists have used the combined mordant and stain, with phosphomolybdic and phosphotungstic, and have found it more effective than either alone, I (22) adopted the idea of using both and it worked very nicely Basically, these methods color the cytoplasm first with some acid anilin dye, then the tissues are exposed to phosphotungstic or phosphomolybdic acid, either mixed with anilin blue or followed by anilin blue It can be done either way

Bauer Does it make any difference?

Lillie It does, but so also do many other details and it is rather hard to evaluate just where the differences are

Of all the variants of the Mallory staining technique, probably the most selective and most conclusive for connective tissue fibrils is the method of Heidenhain (23) This involves staining first with azocarmine, which was misnamed "azan" and hence the name of "azan technique" Azocarmine is an acid anilin dye that is rather purely soluble in water It is dissolved in a dilute acetic acid solution, as a rule Although Heidenhain simply said the solutions were to be well acidified, his followers later specified the amounts of acetic acid to put into the mixture After treatment with the acid dye to stain cytoplasm and muscle, the sections are soaked for a half-hour to three hours in 5 per cent phosphotungstic acid and then stained one to three hours in an anilin blue and orange G mixture containing perhaps 3 to 4 per cent acetic acid The per cent of acetic acid varies There is a tendency, which works quite well, to prolong the time interval and diminish the concentration of both the dye and the acid Apparently, it tends to stain other things less and connective tissue fibrils just as intensively

Porter The collagen swells in that mixture, doesn't it, under the influence of acetic acid?

Lillie: The collagen, in this instance, has been previously treated, preferably with a saturated solution of mercuric chloride containing acetic acid and potassium dichromate, and it is pretty well incapable of swelling except under very drastic treatment

Porter: Does the acetic acid allow for better penetration of the dye?

Lillie: That is the general idea, and it also influences the pH level

Bauer: Perhaps that is the explanation of the wavy appearance of the collagen fibers in sections

Lillie: The wavy appearance was observed in the teased preparation days before staining and cannot be attributed to treatment artifacts, I believe With nothing done to it, a spread of mesentery immersed in physiologic saline shows the wavy fibrils, mixed with the straight, branching, elastic fibrils

Bauer: Wavy fibrils that yield gelatins?

Lillie: Yes When they are raw, they will swell in acetic acid When they have been denatured by various chemical treatments, called fixation, their behavior is modified, understandably.

The other great group, after the Mallory anilin blue and the Heidenhain, is the Masson (7) trichrome method. Newer versions of the original Masson methods vary principally in the substitution of different acid anilin dyes for the background stain. Masson used either anilin blue or light green to stain the connective tissue fibrils, and the later differences are a matter of color choice rather than of innate selectivity The Masson methods are less intense and less total in their selection of connective tissue fibrils than the Heidenhain variants, and they are less effective than some of the better picric acid mixtures for staining all the connective tissue However, they work quite well for routine use, and they are quite duplicable.

In the last decade, there have been a number of single solution stains, such as Ladewig's (24), Shorr's (25), Papanicolaou's (26, 27, 28), and the methods of Cason (29) and Gomori (30) which appeared in 1950 The Shorr and Papanicolaou methods have been used almost entirely for other purposes than the staining of connective tissue, and I think we can forget about them in the present connection. Gomori found that one-solution trichrome mixtures could be made from blue or green acid triphenylmethane or diphenylnaphthylmethane dyes to stain collagen with red sulfonated azo or disazo dyes as plasma stains, acetic and phosphotungstic or phosphomolybdic acids Phosphotungstic acid, he found, tended to intensify the background or plasma stains, and phosphomolybdic, the fiber stain. Alcohol weakened the plasma stain

ings, he made specific recommendations as to what dyes ...

amounts of each to use I have tried them and, like other fast green methods of the Masson type, they give a rather diffuse and incomplete picture of a more finely fibrillar collagen The short cuts do not give the results that the sequence methods do, that is often true in histology, for *we have not yet learned to limit specifically the reactions of a number* of reagents so that they will pick up just the things that we want to show Progress is being made, but sequence methods work better at this time.

Masson developed methods in which he modified the picric variety

acid dye to color cytoplasm and muscle, then wash off the excess acetic acid solution, which can be done with water The acetic acid apparently takes the dye out of the blood plasma, and probably out of the connective tissue fibrils, while leaving it in the cytoplasm and muscle Then one exposes to the picric acid mixture with an acid dye, usually triphenylmethane of the type of acid fuchsin, anilin blue, fast green FCF, or wool green S which is a diphenylnaphthene dye It does not seem to make a great deal of difference which is used They are all sulfonated dyes on that general type of nucleus and they are aqueous solutions Some of them give pH levels around 3; I have not measured all of them, but acid fuchsin does, by itself Actually, acid fuchsin is a disodium salt of a trisulfonic acid, and it is thought by the dye chemists that the extra sulfonic acid group hooks back into one of the amino groups, forming a closed ring However, it seems to be at least partly available to furnish H ion

I have modified this procedure of Masson's He used metanil yellow, which is a deeper yellow than picric acid, acid fuchsin mixtures I used a deeper scarlet which gave us a red background, and a picro-anilin blue which gave blue connective tissue, and it worked very well There are also other variants One of the prettiest is using a fast green with a more or less traditional picric fuchsin, with an increased amount of acid fuchsin in it It gives gray-green, yellowish-green muscle and cytoplasm background, contrasting vividly with a rather completely demonstrated deep red connective tissue

At this point in our investigations, we found that acidification of the picric acid, acid fuchsin mixtures increased their selectivity, and further exploration of this effect led to the discovery that triphenylmethane sulfonic acid dyes become quite selective collagen stains at low pH levels, regardless of the source of H ion, whether it is phosphotungstic, phosphomolybdic, or hydrochloric acid Acetic acid does not give enough H ion by itself in any reasonable concentration

Meyer What pH is this?

Lillie: A pH below 2, preferably between 1 and 1.5, will give quite selective collagen stains It is roughly what is obtained by mixing decinormal hydrochloric acid as a solvent for acid fuchsin. I have not used that particular mixture, but I have used others similar to it We developed a couple of hydrochloric acid methods which were included in the textbook I published a few years ago They are of considerable theoretical interest but I think they are inferior from the point of view of practicality to the better picric mixtures, and perhaps to the phosphotungstic and phosphomolybdic mixtures as well

azodyes that will do the same thing There is naphthol blue black, for instance, a disazo dye, which has, I think, two or three sulfonic acid groups in it and which gives a very intense color. It seems to be selective when used in a quite acid solution. This suggests that it is something of the same nature as the isoelectric point type of staining which has been used a good deal with methylene blue. At certain pH levels, dye is taken up, that is, the basic dye is taken up, by a decreasing number of structures Acid fuchsin is taken up by a great many of the tissues at higher pH levels As the pH level is diminished, it apparently becomes more selective for collagen and reticulum Just what chemical peculiarity this is based on, we do not yet know. We do know that gelatin, which has been denatured by treatment with formaldehyde to render it insoluble in water, still yields this reaction of staining red with Van Gieson's picric-acid fuchsin, blue with Mallory's or Masson's anilin blue stains, and so forth

In attempting to see whether or not I could get anything from peptic digests of gelatin, I ran into some major problems. If done in solution, nothing is seen I tried to precipitate the peptides with phosphomolybdic acid, but I was not at all happy about the spot-test results on the precipitates It could probably be said that there was a certain amount of material which selected the red dye out of the picric acid mixture remaining on the glass slide, where the attempt was made to fasten the precipitate of polypeptide with phosphomolybdic acid, but it was not what I would call convincing, and I won't make any point for it.

I am, in essence, saying that I consider all of these acid anilin dye methods: the phosphotungstic, the picric, the hydrochloric mixtures, as essentially depending upon the same principle of selection from highly acid solution of these dyes by the connective tissue fibril

Porter: What do you think the acidification means in the staining procedure?

Lillie: I have not really figured out what it came down to in that protein molecule

Porter: What happens if fresh tissue is put in a mixture of this sort, without fixation?

Lillie: That would be interesting to try I have not done it

Meyer: Dempsey and Singer attempted to characterize proteins by the amount of dye bound at the isoelectric point of the protein. You are far from the isoelectric point of collagen

Lillie. Unless it has a second one

György One chemical compound cannot have two isoelectric points

Lillie We think of the point at which tribasic acids show a rapid shift of pH on minute additions of acid or alkali somewhat as one might think of a sort of isoelectric point for potassium acid phosphate. We get a pronounced buffer action below that

Meyer: But those are not isoelectric points.

Lillie. Dissociable groups give a certain isoelectric point at one of their dissociable levels, but if you can obtain an additional H ion out of some compound, it does not dissociate until a much lower pH

Meyer A great deal of work has been done on the titration of proteins, especially of wool, by Steinhardt (31) with various anions at varying levels of pH The concentration of anion combining with the protein corresponds to the basic amino groups of the protein down to certain pH values At very low pH values, the uptake of anion is greater than corresponds to the basic amino groups and varies with the type of anion I think you might expect similar effects with the dyes

Lillie You are thinking, perhaps, of a displacement of the sodium from its sulfonic acid position and a combination of the dyestuff with amino groups of tissue, the sulfonic acid acting as a uniting force with the tissue base

Meyer: The proteins in a tissue obviously form very complex systems, in which salt formation and solubility of the complexes play an important role, especially after treatment of the tissues with fixatives. I don't think anybody knows what happens to the structures then

Bennett What Dr Lillie has said points up the difficulties Morphologists over the years have described results and made interpretations based on trial-and error experiments in staining tissue after it has been denatured by harsh fixatives Certain color reactions develop, which are consistent and repeatable These reactions have been terribly important and useful in the identification of cells and intercellular substances However, it is exceedingly difficult to measure many of these reactions in terms of chemical linkages or as precise chemical reactions

Lillie We have not modified greatly the groups that react with these particular dyestuffs by the variety of fixing procedures which we usually use We do speak of some fixing methods being better than others but that does not mean that even those regarded as the poorest do not also give at least some other reaction

Robb-Smith. Must we not admit that the material which stains in

Lillie: Yes, we have modified it chemically.

Robb-Smith: Although it is perfectly fair to say that it is the same material

Lillie: It is fixed collagen, if you like.

Gyorgy: It cannot be the same material.

Robb-Smith: No, but it is analogous.

Lillie: It is not chemically the same substance.

Ragan: It is derived from the same material.

Meyer: I wonder how far we deviate now from your definition of collagen How certain is it that the substances which are stained are identical with those defined by you previously as swelling in acetic acid and giving gelatin on boiling?

Lillie: We must return to the concept that collagen is still a morphologic, as well as a chemical, entity.

Perhaps it would be best to postpone further discussion until I have completed somewhat more of my presentation In 1905, Maresch (32) used the Bielschowsky silver method on some liver and discovered that the reticulum, as described by the anatomist, Mall, and others, was blackened After that, an extended series of papers appeared in considerable number in the next four years, dealing with the exploration of the entire human and animal anatomy to find out where there was reticulum which could be blackened with silver and how much of it there was It was the most complete method at that time for finding connective tissue in the organs, and it served a valuable purpose in enhancing the knowledge of the totality and distribution of connective tissue and its relation to parenchyma. Mall's (33, 34) method had digested the parenchyma out in order to see the reticulum, which was destructive to the study of relationships. With the silver method, it ... solid viscera was more strong- ...nd of general tissues. How- ...m and collagen by the acid anilin dye methods, and the apparent conversion of reticulum to collagen in sclerosing processes remained, and a lively controversy arose as to the identity or nonidentity of reticulum and collagen.

Basement membranes of glands and epithelia also became the subject of controversy. As early as 1865, His, according to Von Ebner (35), apparently regarded basement membrane as a condensation of closely felted, reticular fibrils Von Ebner (35), thirty years later, thought of the basement membrane as a hyaline structure, abutting closely on sup-

porting fibrillar reticulum. However, Ruhle (36), Mall, and others, in the pre-silver days, adopted the concept of the closely felted condensation of reticular fibrils with no overlying hyaline membrane The introduction of the silver methods led to the almost complete suppression of the hyaline membrane theory and the dominance of the concept of a condensation of closely felted, argyrophilic, reticular fibrils against en- [illegible] ild mention that Plenck (37) [illegible] line membrane, distinct from [illegible] renal tubules and sebaceous [illegible] Other basement membranes [illegible] which more or less numerous argyrophilic, reticular fibrils were included

Foot (38) was perhaps the principal exponent of the view that collagen and reticulum were somewhat different substances. The concept had been advanced, of course, much earlier by Mall who had found that there were differences in resistance to tryptic and peptic digestion However, the reticulum was also considerably resistant to trypsin, although less so than the collagen, and the difference may have been based largely on the usually smaller fiber size of the reticular fibril as opposed to the collagen fibril, or perhaps I should say bundle in terms of present-day concepts

Foot related that brief extraction with sodium hydroxide destroyed the argyrophilia of reticulum but left collagen stainable by the acid fuchsin of Van Gieson's mixture, while extraction with boiling water destroyed the fuchsinophilia but left the argyrophilia However, the facts were controverted in this case by Guyon and his co-workers (39, 40), and it was left more or less up in the air Lowry's (41) assay method for collagen apparently included both substances, collagen and reticulum, in the final quantitative determination Morrione's (42) coordinated work between the chemical and histologic methods on liver cirrhosis demonstrates that the increase in the amount of collagen, as determined by Lowry's extraction method, correlates more closely with the increase in argyrophilic reticulum than with fibrous, less argyrophilic collagen

Reticulum is more argyrophilic than collagen, but that is not to say that collagen fibers are not argyrophilic, they are In artificial edema, for example, where the subcutaneous tissues are spread apart by artificially introduced fluids, fixed in that state and then treated with silver by any of the approved techniques, one finds individual fibers blackened for certain segments of their length, and, directly contiguous with unblackened portions, coarse fibers may blacken and fine fibers remain unblackened, or fine fibers may blacken more than coarse fibers I do not know what the determining factor is It seems utterly capricious But

certainly I do not think we can adhere to Mallory's view that the silvering is dependent entirely on the fineness of the fibril. He thought that collagen and reticulum were the same substance and assigned the difference in silvering to fiber size, but that is not borne out because of the variability in results which can be achieved When one finds a segment of an individual connective tissue fiber, which can be followed across the field of the microscope, solidly black in one part and almost unaffected by the silver in a directly contiguous portion, it cannot be explained The silvering is something which has not yet approached the dignity of a chemical reaction because it is inconsistent, and the multiplicity of silver methods which have been introduced for the silvering of reticulum bears ample testimony to their unreliability.

Porter. Can we account for the difference in the behavior of reticulum and collagen fibers in this respect; that is, if we accept the reticulum fiber as a very fine collagen fiber—

Lillie I don't think even that has been conclusively demonstrated

Porter. Will you accept the notion that, in collagen, there is a unit or fibril?

Lillie. Oh, yes

Porter· It is conceivable, then, that all collagens and reticulins are made up of these unit fibers

Lillie· It has not been demonstrated, as far as I have read, for what I will accept as reticulum

Porter. I see Well, connective tissue fibers at the limits of light resolution are usually called reticulum fibers

Lillie. Reticulum, I think, was defined originally by Mall (33) as the framework tissue of the solid parenchymatous organs. It does not participate, according to my present notion, in the loose connective tissues of the subcutis, for instance Consequently, Gross (43), with his demonstration of the finer and coarser fibrils showing the same periodicity, offers no proof I do not say that reticulum does not have the same structure I simply say that I have not yet seen it demonstrated that liver reticulum or renal reticulum has the same structure as subcutaneous collagen

Porter. I have seen many sections of a variety of tissues in the electron microscope, and I have yet to see anything that is not collagen-like in its periodicity. Even the finest fibrils identifiable in the intercellular spaces in tissues have that periodicity If we allow ourselves to accept that as a criterion of collagen or collagen-like fibers, then reticulum and collagen are identical

Lillie Or similar

Porter All right

Lillie: That is, we have already postulated a number of collagens.

Porter: I am not sure that my thinking is straight in this regard but it seems to me that the following questioning is reasonable When the reticulum fiber is blackened, a certain amount of silver is deposited on its surface; would the same amount of silver per unit area of fiber make a much larger fiber appear black? In other words, may not the density of the smaller fiber result from the proximity of the two margins and the consequent optical blending of the silver along these margins?

Lillie: That is a very nice argument which is tenable on the grounds of logic, but when one looks over a series of observations, it just doesn't seem to be; one sometimes finds black larger fibrils and unblackened smaller ones

Porter: I will grant that my experience with silver staining is very limited

Lillie My experience with silver has been long and disappointing

Porter As a rule, however, is it not true that the larger collagen bundles do not show as black as the reticulum bundles? That is the basis for the distinction, actually

Lillie Yes, I think there is more argyrophilia in what we have called reticulum

Porter But could you conclude that the same amount of silver is deposited per unit area of larger fiber as opposed to smaller?

Lillie I don't even necessarily concede that the silver is deposited on the surface, which is part of the logic of that argument

Porter That is true I am assuming that it is

Wyckoff. Those are things which could now be determined An answer could be given with the aid of the electron microscope

Gyorgy: What is your argument that reticulum and collagen fibers are not similar?

Lillie: Collagen and reticulum are certainly, histochemically, essentially similar throughout, except for this one thing about argyrophilia, which has been argued back and forth for years

György But you yourself admit it is capricious, therefore, you have no facts

Lillie: I have no faith in the discrimination

György And no fact.

Lillie No firmly established fact, no I have shown that there is a variability, and it is not necessarily the finer or the so-called reticular fibril which blackens selectively

György. Whereas the periodicity speaks very much in favor of similarity?

Lillie I should say so, yes

I shall go on now to periodic acid It was shown in a series of papers by McManus (41, 45 46, 47) and Hotchkiss (48) and myself (49,

50) that the application of the periodic acid oxidation followed by application of the Schiff sulfite leucofuchsin aldehyde reagent would demonstrate some of these fibrous substances in the red-purple color of the aldehyde reaction Both collagen and reticulum are demonstrated by this procedure when the techniques are appropriate. The Hotchkiss procedure may often fail to show them because Hotchkiss interposes, after the periodic oxidation, a rinse with sodium thiosulfate, which is slightly acidulated with hydrochloric acid and, in our hands, usually promptly smells of sulfur dioxide It is known that sulfite, applied after periodic acid, will often render collagen Schiff-negative (51) One can overcome that by prolonging the exposure to Schiff reagent; that is, the sulfite combination with aldehyde is displaceable

Various other reagents may be used, as well as Schiff reagent, for demonstration of the polyaldehydes created by periodic acid oxidation of cisglycols or cisamino alcohols I believe the amines may be secondary, that is, chromaffin, which is thought to be the chromic precipitation product of adrenalin, reacts quite brilliantly.

The periodic Schiff procedure did demonstrate hyaline basement membranes with greater intensity than it demonstrated reticulum or collagen. This was shown by Gersh and Catchpole (52) in 1949, but when uncounterstained, the color of the basement membrane was about the same as that of collagen or reticulum There are intensity variations, but no essential discrimination In attempting to see if there were any mucins that did not react to periodic, we found that counterstaining with blue basic anilin dyes would change the red-purple of mucin toward the violet; that is, there is uptake of the blue dye on top of the red. When we used a picric acid counterstain for cytoplasm, the color of collagen was changed from red-purple to orange. That is true of most collagens, except that of cornea which remains red which may be due to the content in cornea of metachromatic substances, chondroitin, hyaluronate, or something of that sort The idea therefore occurred to me to try one of the connective tissue stains that would contrast in color with the red-purple of the periodic Picromethyl blue was used as a counterstain, and one of our first trials produced amazing results Basement membranes appeared deep red in the kidney, the reticulum between the tubules and around the blood vessels in deep blue, with no intergrading of color. There was a certain amount of adjustment of the proportions of methyl blue and picric acid necessary, but when the ideal proportions were worked out, we obtained consistent results Increasing of the methyl blue to tenfold concentration did not reverse the color of the substances which had remained red. There was discrimination of collagen and reticulum which changed their color to blue; hence, the term that we have introduced, "allochrome," from ἀλλόχροος, changing in color (53).

This method shows, for example in Figure 1, that the ring fibers about the splenic sinuses are composed of a material which stains like that of the basement membrane of the kidney, whereas the pulp fibrils color blue, as does renal reticulum and most of the trabecular material, with greenish-yellow muscle, blue connective tissue fibrils, and a few interspersed, red, sheath-like fibrils They look like little circles of red surrounding bundles of blue fibers In the coarse connective tissue surrounding arteries in the kidneys, there are also little rings of red around large bundles of blue Sarcolemma, in striated muscles, shows a red layer next to the muscle sarcoplasm, with blue fibrils running in between the muscle bundles and almost onto the surface of the red membrane It is more apparent following tryptic digestion when the red layer of muscle swells somewhat, then one sees more plainly the blue fibrils running over the more or less wrinkled envelopes. The blue fibrils are argyrophilic, but the sarcolemma proper is apparently nonargyrophilic

In dietary liver cirrhosis of rats, the fibrous material showed up in blue, the ceroid pigment in red, and there is a little glycogen which also stains red, as is true with periodic acid

Porter Will you review the procedure again, please?

Lillie Periodic Schiff reaction followed by iron hematoxylin and picromethyl blue

In torular meningitis, the torula show up in red, the myelin in more or less red color

Figure 2 is of a more or less normal kidney It can be seen quite plainly in some areas where the tubules are a little more widely separated that there is blue fibrillar material between red membranes surrounding the epithelium The epithelium is rather pale in this stain The framework of the glomerulus is almost entirely of the basement membrane or red material

In the renal medulla, the blue connective tissue can be seen between the red basement membranes In pyelonephritis, hyaline casts in the tubules are stained deep red The amount of connective tissue increase can be rather plainly shown in blue, and collapsed glomeruli with blue crescent-like connective tissue formations between Bowman's capsule and the glomerular tuft may be seen Amyloid colors in a lighter purple Apparently the basement membrane of the glomerulus frays right into the amyloid

Bennett If you had superimposed a silver impregnation, what would have happened, Dr Lillie?

Lillie What happens if the silver impregnation is applied before and then toned with gold so as to render the impregnation insusceptible to the oxidation? What we did actually was to make a considerable

series of reticulum preparations, both by one of my own and by Wilder's method, and then examine the slides for the best reticulum impregnations that we could find. Those sections were demounted and put through the periodic acid method. Then the basement membrane shows up as clear red hyaline lamina in between the epithelium and the black reticulum. We did that on a considerable number of preparations, chiefly of the kidney because the basement membrane is easiest to see there Basement membrane is also found in the pancreas but it is very thin in comparison to that in the kidney.

Bennett. In other words, the material which reacts with periodic acid Schiff is not silvered Does it encompass the silvered material or is it just on one side?

Lillie: We have had very poor success in silvering the stroma of the glomeruli. In staining two glomeruli, side by side, in a case of renal tuberculosis, where one of the glomeruli has undergone involution and scarring and collapse, the red material would be the collapsed glomerular stroma, the blue crescent around it the ingrown connective tissue. With the silvering, it would still be found that the red next to the epithelium was the tubule, but if the full sequence of the periodic and picromethyl blue were done, a quite variable mixture of black and blue fibrils would be found between the tubules. If the silvering has been fully adequate, there is practically no blue. If some of the reticulum of the kidney has failed to blacken silver, then the unreacted fibrils take up the blue from the picromethyl blue. In sections of Kimmelstiel-Wilson diabetic kidney, much the same is seen. The globular material in the glomeruli seems to be related to the substance that makes up the basement membrane in so far as this reaction goes

Bennett· It is a hyaline substance, whatever that is?

Lillie. Yes

Bennett Does it ever take any of the other stains that we accept as being stains for collagen?

Lillie· My recollection is a little hazy on that. It behaves like amyloid and like basement membrane with this technique and not like collagen.

Robb-Smith. What do ordinary, classical collagen fibrils in the dermis, for instance, look like with this method?

Lillie: Blue.

György. Elastic fibers are stained?

Lillie· Elastic fibers are not stained. We ran control preparations with both orcein and the Weigert resorcin fuchsin method on the same preparations. The distribution of fibrils stained by those methods did not correspond to either of the types seeen

Bennett: Do you interpret the blue material as being collagen?

Lillie Yes.

FIGURE 1 Spleen X720 Lattice fibers about sinuses in red, reticulum and connective tissue in blue, erythrocytes bright yellow, nuclei brown

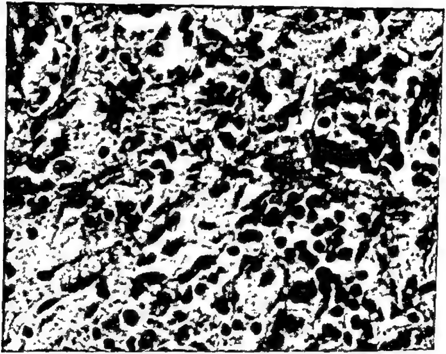

FIGURE 2 Kidney X325 Basement membranes of tubules and glomerular vessels in red, reticulum in blue, basal cytoplasm in greenish yellow, brush border in purplish red, plasma in orange, erythrocytes in yellow

Bennett: How do you interpret the hyaline material which stains intensely red?

Lillie I have made no detailed survey of the other reactions of these various forms of hyaline material It should be done, and there will undoubtedly be differences shown They are alike in possessing linkages which give rise to this one reaction That does not mean they are identical It does not mean that they are necessarily mucopolysaccharide.

Porter. It doesn't describe them as mucoprotein?

Lillie. If they are mucoproteins, so is adrenalin

Ragan: Is your material formalin-fixed?

Lillie: Yes

György. Did you find any difference between the reticular fibers in collapsed necrotic liver and the cirrhotic fibers?

Lillie: In both the normal liver and the cirrhotic liver, we find no red-sustaining basement membrane. It differs from all other organs in that respect

György. They are blue?

Lillie Yes.

György. In hepatitis, is the same blue color found as in cirrhosis?

Lillie. Yes And in cirrhosis, we find the trabecular bands made up of blue-staining fibers

György: But you do not conclude from this that they are identical?

Lillie. Not necessarily There may be other points of difference shown In the liver we do find basement membranes about the bile ducts stained in red, and we also find mucin in bile duct goblet cells stained red Arteries will show the same red stroma in the inner portion of their walls that they do elsewhere, but the liver parenchyma itself seems to be composed entirely of the blue-staining reticular fibrils If this indicates, as I think it does, that there is no true basement membrane in the liver, it would apparently put the liver cells in much closer contact with the circulation than are the renal epithelial cells with their fairly thick, relatively speaking, basement membrane between the supporting connective tissue and the epithelium

György The ground substance is stained?

Lillie The ground substance Since we are staining the connective tissue fibrils, the collagen fibrils, in blue, we will sometimes see in umbilical cord, in the rather wide lymphatic spaces, small coagula which stain red They are metachromatic They are destroyed by hyaluronidase which has been boiled in acetic acid, which will no longer depolymerize hyaluronidase *in vitro*

Meyer Do you demonstrate destruction by the fact that they do not stain any more with the periodic acid Schiff reaction?

Lillie: No, we prefer, in this instance, to use metachromatic dyes

Bauer: What is the source of your hyaluronidase?

Lillie: Bull testis preparations from Schering and Wyeth, which are [illegible] hyaluronidase changes the [illegible] olishes the metachromatic staining of cartilage matrix.

Gyorgy: How much hyaluronidase did you have in your solution? Is it possible that the boiled hyaluronidase is adsorbed to the material and thereby causes a change in staining quality?

Lillie: I do not know. There was abolition of the metachromasia.

Meyer: Dr Lillie said the disappearance of the metachromatic staining was due to a chondromucinase. The suffix "ase" obviously designates the agent as an enzyme. *There is no proof whatever that an enzymatic action is involved, either with the testicular preparation or with the malt diastase* We have tested both enzymes for sulfatase activity, using chondroitin sulfates as substrates Testicular hyaluronidase hydrolyzes chondroitin sulfate of cartilage into low molecular fragments which are still sulfated. The same enzyme also hydrolyzes chondroitin sulfate from umbilical cord (which we termed chondroitin sulfate C) *without splitting off sulfate.*

Lillie: We tried to carry out the histochemical digestion in the presence of barium salts to see if there would be any demonstrable barium sulfate crystals, and while there was destruction of the metachromasia, no sulfate crystals were found.

Meyer: I would agree with Dr. Gyorgy's explanation. I also doubt that there is any enzymatic reaction here. The whole literature on metachromasia is confusing You probably saw a recent paper by Levine and Schubert (54) on metachromasy which to a large extent is a confirmation of older work by Bungenberg de Jong (55) From this work, it appears that the basic dye is bound to an anionic complex by both polar and van der Waals' bonds. The polar binding can be influenced by neutral salts, the van der Waals by alcohol, for example. If the dye is to be bound, it must replace some other anion which is combined with the acidic complexes in the tissues.

Very large concentrations of hyaluronidase were used in your experiments, similar to your experiments with malt diastase, boiled or unboiled I suspect the explanation is the same for both types of agents, namely, that some protein present in these enzymes interfered with the binding of the dye to acidic components in the tissue. I think you are not permitted to conclude more from your experiments

Lillie: The buffer system alone does not do it.

Meyer: I did not think it was the buffer system

Lillie: I think it is true that the capacity to reduce metachromasia in umbilical cord does not parallel the turbidity reduction unit titer of a hyaluronidase preparation, that is, when the concentrations used are varied and we try to reach some sort of an evaluation by the concentration-time relationship that it takes to clean out the metachromatic material completely, we find that different lots of enzyme, varying as much as a hundredfold in their enzyme titer, as determined by the turbidity reduction technique, will show only tenfold variation in their capacity to digest the connective tissue mucin.

Meyer: Connective tissue mucin as demonstrated by metachromasia?

Lillie Yes, that's right

Meyer: I object to the use of the word "digest." Your observations are undoubtedly correct Your interpretation I do not accept You can say that you change the capacity of this material to combine with the dye But to call that digestion implies a mechanism which has not been proven

Lillie The observations were, as you may have guessed, more or less incidental to other work that was in progress, and they were deemed worthy of putting on record in order to stimulate some biochemical in-

grounds on which you discriminate between digestion and the denaturation which has occurred

Meyer: I shall not attempt to explain your experiments I cannot. I do not know, nor do I believe that anyone else knows either

György: You said it was enzymatic action, and our objection is to that statement because it can be explained by other means

Meyer: I believe Lison, and also Bungenberg, demonstrated that mono-, di- and tri-valent ions will abolish metachromasia

Lillie. At what levels, though?

Meyer: The concentrations are 10^{-1} to 10^{-3} molar One would certainly not call the action of NaCl a digestion

Lillie No, but the term digestion can be used in relation to the effect of hydrochloric acid on proteins

Meyer Hydrochloric acid at room temperature? No, I certainly would not use the term digestion in that case

György Hydrochloric acid would still not be an enzyme.

Lillie Oh, no

György: But you infer that it has an enzymatic action

Meyer Also, hydrochloric acid with most proteins is quite stable;

a lot of proteins are quite stable with hydrochloric acid at the concentrations of tenth normal which you use

Lillie: In this instance, yes.

Meyer: You may even heat some proteins with 0.1 N HCl without destruction of the protein.

Robb-Smith: I wonder if we could come back for a moment to the periodic acid-Schiff reaction itself? If I understood Dr. Lillie right, he said that classical collagen, such as he was discussing earlier, provided there was not too much sulfite, did stain red?

Lillie: Yes

Robb-Smith: On the other hand, by this technique, where you are using a counterstain, you get no red staining of collagen or fine reticular fibers

Lillie. That's right

Robb-Smith: What do you feel this means, considering that, if you use the ordinary PAS, classical collagen does not stain red; that is, it stains yellow, whereas your fine reticulin fibers, possibly with basement membrane, do stain red?

Lillie: McManus and I have both objected to the classical Hotchkiss on the ground that it introduces what is probably a partial sulfite blockade after the periodic oxidation, thiosulfate, or the sulfate step is omitted It has been our experience that the collagen does stain red

Meyer. May I ask whether you have tried the commercial hide powder, the standard hide powder which is sold to evaluate tanning agents?

Lillie: No I do not know of it

Meyer: It is collagen, and I think the experiment would be valuable because it is certain that it does not contain any of the ground substances, or the substances which are thought to act primarily with periodic acid-Schiff reagent

Gyorgy. Does it contain sulfate?

Meyer: No There is no chondroitin sulfate or other mucopolysaccharide in this material, at least, it contains no hexosamine in demonstrable quantities. It may be possible that periodate will oxidize some of the amino acid groups of collagen, but I should like to see it demonstrated I remember two years ago Dr. Gertrude E. Perlmann mentioned that insulin was oxidized by periodic acid.

Lillie. But if this reaction of collagen fiber with periodic acid is due to imbibed ground substance, is it not strange that the ground substance itself, which is between the fibers, fails to react and that all of this material is apparently concentrated within the morphologic unit of the fiber?

Meyer. Is it not possible that, by the process of fixation, the ground substance has been deposited onto the collagen fibers?

Lillie The metachromatic material has not migrated. It remains [illegible] between the fibrils

Meyer: On the same sections?

Lillie: Both cannot be done in the same section The next section of the same lot has to be used

Robb-Smith: I should like to come back, Dr Lillie, to the question of why it is that you do not get classical collagen staining with PAS whereas with your uncounterstained methods, when you are avoiding too much sulfate, you do

Lillie Simply because, in the course of periodic oxidation, we have [illegible]

[illegible]

amine an individual fibril by spectroscopy, we would find it still absorbed at 570, probably as much as before

Robb-Smith But surely you would have expected to see it a good deal more purple than it is in your preparations You got a good red if your collagen fibers were in fact a good red before you added your counterstain

Lillie: The red is not as strong as it is in the basement membrane.

Robb-Smith. No, there is a difference That is what I want to get at.

Lillie: There is a difference in intensity

Robb-Smith No matter how you color it?

Lillie: Yes I would say that the explanation of the deposit of the periodic Schiff reaction might well rest on relatively minor quantities of hydroxylysine or threonine in the collagen itself, if they are available for reaction I don't know that that has been worked out I would like very much to have a reaction for hydroxyproline or proline

Holbrook Have any studies of this kind been attempted on unfixed material, and if so, is it at all stainable?

Lillie Gersh did a certain amount with periodic on frozen, dried material I did not discern in his work any vast difference from [illegible] so we have not encumbered ourselves with the much greater [illegible] complications

Meyer: But I thought Gersh said that the collagen fibers [illegible] do not stain with periodic acid

Holbrook. That is what he said last year which is the [illegible] why I asked the question

Lillie He is still using the Hotchkiss procedure.

Meyer I'm sorry, but what is the difference between [illegible] procedure and that of Hotchkiss?

Lillie: We omit any reducing reagent after periodic [illegible]

Ragan: Does collagen take stains such as the Masson and Heidenhain when it is unfixed?

Lillie: Not having tried it, I cannot tell you

Ragan. Such an experiment might explain whether denaturation progresses to the point where collagen is modified. I don't know of any work that has been done with those stains on frozen, dried material

Meyer: It would appear questionable whether your method leads to the production of aldehydes. The leucodye becomes converted to the oxidized dye; this oxidized dye, by adsorption or by a primary valence bond, combines with some group or groups on the fibers. Then you superimpose on that a second stain which also has an affinity to the protein. Is it not possible that you are dealing then with a competition of the different dyes for these fibrous structures? The interpretation as to the production of aldehydes and the formation of Schiff's bases seems to me somewhat risky.

Lillie. The periodic reaction of collagen is readily inhibited, or the Schiff reaction of collagen after periodic oxidation is readily inhibited, by interposition of a bath in sodium bisulfite solution, or by phenylhydrazine or by semicarbazide Dempsey has shown that the Seligman Ashbel agent, naphthoic acid hydrazide, can be used in place of the Schiff reagent to demonstrate periodic produced aldehydes But I don't recall enough of the details to remember whether he mentioned specifically collagen and reticulum. We have other aldehyde methods that have been used to interfere with the Schiff reaction at least.

Meyer: At what pH is periodic oxidation carried out?

Lillie Usually around 2 to 3. Sodium periodate will not work. It is an alkali that does not even oxidize glycogen. I almost missed out on the thing entirely because Dr. Hudson said, "Take some potassium periodate," and forgot to tell me that he had acidified it for oxidizing starch I tried out the alkali periodates and they wouldn't work Then, recollecting our experience in oxidizing methylene blue with bichromate, that it was not successful unless acid were added, we tried adding the acid and then got results. Slightly more than a stoichiometric proportion of acid must be added to the potassium salt

Meyer Gersh digested his slides with saliva or another type of amylase and in that process removed a good deal of material which definitely is oxidized by periodate.

Lillie: Yes, we have done a number of those things as well.

Meyer: There is no difference in the pictures which you see?

Lillie· Such things as I have shown in the Figures have been subjected to digestion with diastase, which is adequate to remove glycogen from heavily laden liver or cartilage completely.

Holbrook: Do you think, Dr. Lillie, that there is any hope for at-

tempting to determine the chemical structure of stained substances by the way in which they stain?

Lillie We may be able to give some indication of affinities or of reactive groupings, but I do not think one could carry through a complete analysis any more than can be done on spot tests The spot testing is a rather parallel procedure

Meyer If I recollect, you conclude that the red material in the pathological kidney glomeruli is collagen which has been dissolved

Lillie No, we are still at the stage of recording reactions and are trying not to draw too many conclusions.

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THE FINE STRUCTURE OF CONNECTIVE TISSUES

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WHAT I PROPOSE to do is to bring up a number of the problems of the fine structure, or ultrafine morphology, of connective tissues that seem to me inadequately solved. At the outset, I should like to say that the questions which can be asked about the morphology of connective tissues have been greatly altered by the development, first, of x-ray diffraction, and, second, by the introduction of electron microscopy (1, 2, 3, 4, 5). With these two tools of research, we can reach down to the macromolecular particles, at least, that are constituents of connective tissue.

X-ray diffraction was the first to be used extensively. It has incomparable value for revealing the atomic arrangement in crystals, but its usefulness to research on connective tissue is now, I think, very much secondary to that of the electron microscope. While collagen in its various forms does give characteristic x-ray diffractions, we are quite certain that collagen is not a true crystal and, as morphologists, we are more interested in the arrangement of molecular particles of collagen than questions involving the arrangement of atoms within its molecule for the time being. In talking about collagen structure on the molecular level, the electron microscope is of more immediate and, I think, wider value. What I have to say has to do entirely with electron microscopy and I shall quite frankly ignore the x-ray diffraction side of the fine structure problem.

Of the various questions to which I should like to call attention, one is, in a sense, the basis for all the others and it is really twofold. What, from the standpoint of their visible fine structure, are the kinds of connective tissue elements which are seen in nature, and what is the relation between them? The answer to the first is rather clear-cut and elicits general agreement; it is that fibrils of two sorts are seen in connective tissue. I should like to illustrate with some Figures, mainly in order that we may be agreed as to what we are talking about. The commonest fibrils are characterized by striations that are *ca* 650 Ångstrom Units apart. They are shown in Figure 3 which is of a fragment of teased ten-

FIGURE 3 A fragment of tendon from rat tail Note the different diameters of the fibrils and the alignment of their 650 Å striations Reprinted, by permission, from Wyckoff, R W G *Electron Microscopy, Technique and Applications* New York Interscience Publishers 1949

don The separations of 650 Å are, of course, those between the bands or ribs seen here Also seen in connective tissue are fibrils which are more or less continuously striated, with about one-third the 650 Å separations Figure 4 is an example of this closer striation It is a bit of teased material from around a muscle, and the banding along each fibril is about 210 Å

Porter Is this material from a young animal, Dr Wyckoff?

Wyckoff No, this was from a mature rabbit It is also true, of course, in line with the work of Orekhovich (6) and that of Highberger, Gross, and Schmitt (7) that we can make differently striated fibers in the laboratory through the interaction of mucoprotein with a collagen solution

FIGURE 4 A fragment of co... all about 210 Å, instead of 650 Å, apart. ... from Pratt, A W, and Wyckoff, R W G · The fine structure ... nective tissue fibrils *Biochim et biophys acta* 5, 166 (1950)

Figure 5 will serve to illustrate this The interstrial distance, that is, from the one thick ridge to the next, is several times the 650 Å of Figure 3 In addition, there is a fine structure within each striation which looks very different from what is seen with collagen.

Porter: Is the larger distance a multiple of 650?

Wyckoff: I am not sure that I know.

Porter: It would be interesting if everything were built up on a 210 or 220 basis, wouldn't it?

Wyckoff: If it were, yes. I think it could be built up on that basis here, but whether there would be any meaning in the result in view of our inaccuracy of measurement, I don't know.

In these fibers, there are considerable variations of detail within a

FIGURE 5 Fibrils of mucoprotein-collagen complex In this preparation there are two minor bands between each of the more pronounced ones These minor elevations are not always apparent

striation Sometimes, as, for instance, at the top of Figure 5, the bands within a striation are almost completely suppressed There are many cases where they are completely suppressed, and others where they are exaggerated to furnish a uniform striation of about one-third the full spacing of this Figure

Mirsky Exactly what is this?

Wyckoff These are fibers which are made by mixing a mucoprotein with dissolved collagen and then dialyzing

Bennett How do they differ from myofibrillae? Is there any difference?

Wyckoff Some of it looks surprisingly like fully contracted muscle

Bennett That is what I was thinking

Wyckoff But this is clearly a superficial similarity, although it is disturbing sometimes

Porter: Did you ever see fibers without anything in between the striae? There is a bit of a suggestion of it in Figure 5

Wyckoff. Yes. I have some photographs in which nothing at all can be seen

Porter Isn't that a little mysterious?

Wyckoff: It must mean that the stuff in the middle is enormously hydrated One can also get, by working at somewhat greater concentrations, this same striated material, not as a fiber. The striations will develop within a drop and there will be a variety of detail within the drop Hence, a fibrous end product is not necessary. As far as I know, this sort of thing does not occur in nature.

Porter: I have seen things of this sort in cultures, except that the periodicity was not as great. It was only about 1100 to 1200.

Bauer Why do you say it does not appear in nature, Dr Wyckoff, when we know that this phenomenon can be produced with concentrations of the material that are encountered in diseased states.

Wyckoff. All I would say, then, is that I don't think we have encountered it in nature.

Bauer. That would be all right.

Porter I could argue on that point, for probably at no time in a diseased state is there the concentration that there is at the beginning of this reaction

Bauer: You have no way of proving that.

Porter Indeed, no, but you have no way of proving your point either

Bauer: I am talking about concentrations that may occur in the blood What concentrations may exist when collagen is in solution, I don't know

Meyer. But you don't dialyze. You don't have an absence of electrolytes in nature

Wyckoff I would say that of the two naturally occurring fibrils, that is, the 650 Å and the 210 Å types, to designate them loosely, those with the 650 Å repetitions certainly seem to be the more common, the more widespread. As you will see from the Figures which follow, the fibrous elements of connective tissue are more often than not so contaminated with the substances in which they are imbedded that their striae are obscured, unless very special steps are taken to clean them The close 210 Å striations, as in Figure 4, are more likely to be covered over by these contaminations, and it would be much easier to miss them than to miss the 650 Å. Thus, at this stage, when there has been a relatively small amount of exploratory work done, it must be said merely that the 650 Å fibers seem to be the more common. This may or may not be the case

There is a striking difference between the two types of fibril the

650 Å fibrils differ widely among themselves in diameter and in the details of their fine structure, whereas the 210 Å striated elements are always, as far as we have seen, relatively small in diameter and, in a single preparation, their individual fibrils are much more uniform in diameter than the 650 Å fibers are likely to be. Figure 3 of 650 Å tendon shows wide variations in the diameters of the individual fibrils, whereas the 210 Å fibrils in Figure 4 are surprisingly of the same diameter, or very nearly so

To turn to the second part of my initial question, it is not easy to establish the exact relationship between these two kinds of connective tissue At last year's Conference, in the work done with tissue cultures of fibroblasts, Dr Porter expounded the view that the continually striated elements are in a sense immature with respect to the 650 Å fibrils, and that they tend to pass over into the 650 Å fibrils with age (2) On the basis of our experience, there can be no doubt but that the 650 Å fibrils are more conspicuous in adult tissue and that, as I think Gross indicated, the 650 Å fibrils themselves are thicker in older tissue than in younger tissue

Porter They seem to increase in size with age, but don't you suppose there is a maximum for each tissue? The collagens are of different widths

Bauer. In different organs

Porter Yes, in different organs

Wyckoff Yes, I would think there is a maximum. But there are several facts that make it uncertain that the 210 Å fibrils are immature with respect to those with 650 Å striations We have not infrequently found bundles of the 210 Å fibrils in tissues of apparently healthy animals

Bauer Adults?

Wyckoff Adult animals, as in Figure 4 which was from an adult rabbit

Meyer That was from a muscle?

Wyckoff. Yes, from the neighborhood of a muscle

Meyer Is there a difference in what tissue you investigate, or is that immaterial?

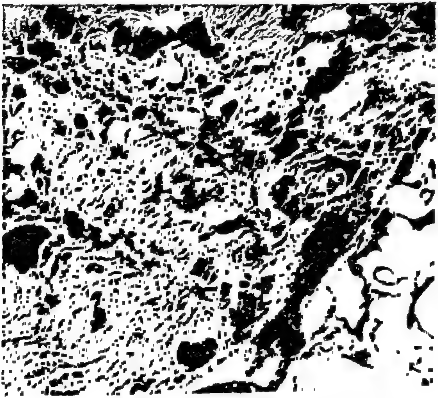
Wyckoff I don't think we know Actually, I should probably admit that I am a little embarrassed to be talking about connective tissue because our experience with it is really more or less incidental to work on other problems The primary business of my laboratory now is with virus diseases, and what I have to say here about connective tissue is in a sense only the fruit of running observations which we have had to make I am interested in the so-called dissolved collagen in its own right, and this is the only aspect of the whole collagen problem to

which we have given what I would call serious study Thus, I haven't experience covering many tissues.

Mirsky: So we know you are not prejudiced

Wyckoff: No, I am not at all prejudiced.

Another point is that fibrils of the 650 Å collagen can appear very promptly, and I think seemingly as a primary product of the activity of fibroblasts. For instance, if chick embryo membrane is inoculated with influenza virus, there is often a very marked thickening of the mesoderm within forty-eight hours. Masses of newly formed fibrils will be found, many of which do show typical 650 Å striations. Such a mass is seen in Figure 6, which is a picture at low magnification of the mesodermal layer of such an infected chorioallantoic membrane



[illegible] mesodermal portion of the [illegible] Wavy [illegible] field [illegible] of the [illegible] picture

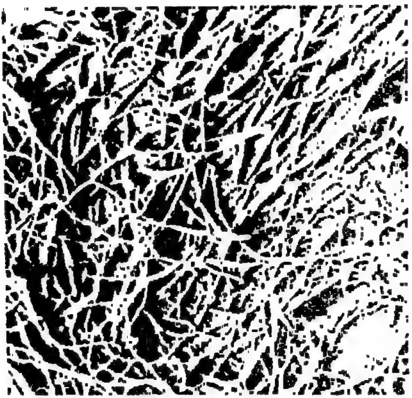


FIGURE 7 A portion of a mass of new connective tissue filaments at a magnification high enough to show their striated character.

The 650 Å striations are definitely discernible in an enlargement (Figure 7)

Porter But the 210 can also be seen

Wyckoff. Yes, I think so

Porter: We should bear in mind that chorioallantoic membrane is a relatively well-differentiated tissue and some of this could be there before you put the virus in

Wyckoff Yes, although I think very little of this is old collagen because of the tremendous proliferation that has occurred This is right in the middle of that region of great proliferation

Porter Yes I am inclined at the moment to interpret some of the 210 fibers we thought of as being characteristic of the conditions in tissue culture as being a product of those conditions rather than a stage in development

FIGURE 8 Filaments of connective tissue seemingly developing *the surface of the fibroblast, part of which fills the center and bottom* portion of the photograph They are striated like those in Figure 7.

Meyer: Had the section in Figure 7 been treated before with lime water or trypsin?

Wyckoff. Only by fixation We have had occasion to look at quite a few sections of connective tissue in which these fibrils appear quite close to the individual cells In Figure 8 it may be possible to see that the material adjacent to the cell is striated This is embryonic chicken connective tissue, not from the chorioallantoic membrane. I believe that these fibers actually are produced along the border of the fibroblast, and that they have been thus produced recently.

Porter: Where would the fibroblast be, do you think?

Wyckoff: At the left is part of the fibroblast. And elsewhere, not shown in this Figure, are other masses of fibers that seem to be coming off its surface.

Another observation which I think bears on the relation between the 210 Å and the 650 Å collagen is the fact that both can be produced from the same so-called solution of collagen by varying the physicochemical conditions of precipitation. I shall revert to that later

The relation between these two forms of collagen has the most direct bearing possible on everything that the electron microscope will disclose about both the development and the repair processes that involve connective tissue It is a most important point, but it is one upon which we need a great many more observations than we have at the present time.

Dempsey: You have mentioned only the collagenous and reticulum fibers of connective tissues Have you any observations on elastic fibers?

Wyckoff: None whatsoever We have done no work with elastic tissue

Dempsey: And there is nothing in your sections which is recognizable as an elastic fiber?

Wyckoff: No, but we don't happen to have looked at material that would be rich in elastic fibers

Lillie: Has any electron microscopy been done on material that has been treated by one of the amine silver processes to impregnate reticulum?

Wyckoff: To the best of my knowledge, no

Dempsey: Gross has some information on reticulum fibers which have been silvered He showed that the silver deposits on fibers of a certain diameter, and that less intense silvering occurs in smaller fibers However, I believe he has not made any serious study of it, nor published anything on the subject

Porter: It is quite interesting to play with the notion that there may be a fundamental periodicity in biological fibers Fibrin has a periodicity of 220 Å or 240 Å, and it is 220 Å here in collagen There is something of the same sort in muscles, if I am not mistaken

Wyckoff: This periodicity is surprisingly uniform in all fibrillar material

Gyorgy: What does periodicity mean?

Porter: It reflects or describes the macromolecular structure of the fiber

Wyckoff: Or do you mean what do we mean by 'periodicity'?

György: No I know that But what is the substrate? Why do you get this?

Wyckoff: It must be a reflection of a regular repetitiveness of some sort within the constituent molecules.

Meyer: Couldn't you say that you have in these areas a higher concentration or a greater density of material?

György: Yes, of nonvolatile material.

Wyckoff: This is surely true from the very fact that striations are seen

Porter: And the fact that the periodicity is related to an elevation means that it is a concentration of nonvolatile material, not simply a concentration of atoms of greater density to electrons

Dempsey: How much variability is there in the periodicity of fibers such as this?

Wyckoff. It is very difficult to say because there is always the problem of shrinkage and expansion on the substrate.

Dempsey: That is what I am really asking about. Can one experimentally, by shrinking or by any other kind of treatment, deliberately alter the periodicity? Is it controllable, or is it something which is variable from fiber to fiber?

Wyckoff: If a specimen is prepared from a stretched fiber, it may show a greatly increased periodicity. However, if there is a local break somewhere, stresses are set up in the preparation which produce stretching in one direction and shrinkage in another It is such distortions that set the real limit to the accuracy with which these *striations* can be measured.

Mirsky. But do you not have a control in terms of the relationships between these intervals? In other words, can't you control the influence of stretching or shrinking in terms of the relationship of the periodicity to some other measureable factor in the fiber?

Fischel: At the two intervals?

Mirsky. Yes, or some such thing as that

Meyer: Actually, I believe part of this question has been answered by Gross and Schmitt who published electron microscope pictures of impressions of wet material.

Porter. Wet fibers?

Meyer Yes, and the impressions have the same periodicity. Does that not answer your question?

Dempsey. Not exactly, because I wasn't asking whether the period was artificial or spurious in such preparations as this I was curious because collagen ordinarily is regarded as an inextensible substance, and yet. I was under the impression that the periods could be greatly extended by stretching

Wyckoff. By distortion, yes There exist pictures, especially those that were made, I think, without any substrate at all, in which fibers

are drawn out like a rubber band. The extension may be six or eight times

Dempsey: Well, this is a dilemma, an unstretchable substance which stretches.

Bauer: The regularity with which this periodicity is seen is also interesting, despite what you say

Dempsey: What is seen, though, is a statistical periodicity. If the period is statistically charted in a frequency curve, the 650 Å periodicity is the most frequently encountered, but the curve is bell-shaped There are many individual fibers which have either longer or shorter periods

Wyckoff: But I do say that one doesn't know if those are really variations in spacing or the result of distortions in the sample.

Dempsey: That's right That is what I was trying to get at

Porter: I believe it could be safely said that it is distortion in the sample because in sectioned material, where the water is replaced by various media, the periodicity is amazingly precise and constant.

Dempsey: There is less variation

Fischel: In such stretched fibers as you mentioned, Dr. Wyckoff, is the stretching uniformly distributed between the dense and rare segment?

Wyckoff: No, it is mostly in the less dense portion

Fischel: Then you can't arrive at any ratio between total period to the period of dense segmentation?

Wyckoff: No, I wouldn't say so.

Dempsey: I dislike to belabor this point, but in the so-called stretched fibers, the ones with the long period, has any investigation been made of the birefringence? Is the orientation, as detected with the polarizing microscope, changed in any way in such a stretched fiber?

Wyckoff: I don't know that

Porter: Stretched fibers are extremely small in diameter I don't think they could be studied in that way.

Wyckoff: I agree, but an experiment bearing on Dr Dempsey's question could be set up with tendon, that is, tendon could be put under tension and its birefringence examined I just don't know if anything of that sort has been done

Porter: Hasn't somebody taken x-ray diffractions of stretched fibers?

Wyckoff: I expect someone has, but I don't know

Porter: It is felt, is it not, that there is some turnover in collagen tissue, that is, a turnover of collagen in the life of the tissue? Or is there the same collagen at the end of life that was started with? Do the pathologists have something on that?

Bauer: The pathologists have some evidence that there is some turnover in collagen, but I can't give the reference.

Porter: In almost any sample of collagen that one would take, then, one would expect to find some young fibrils mixed in with the older

Robb-Smith: Using isotopes to study this, a very slow turnover is found, I think

Ragan: Last year at this Conference, data were presented to show that reticulin was measured as well by Lowry's (8) method. According to Sprinson and Rittenberg's (9) work, tendon collagen probably has a turnover which is very slow.

Meyer: It could be suspected that there is some turnover from the fact that the collagen fiber, at least, can be attacked from the end There has to be an end to each individual molecule or fiber or aggregate, and it can be attacked by enzymes.

Lillie: In the chronological development of a number of disease processes, there is apparently a great increase in the amount of collagen, and then there may ensue a very considerable decrease. It could be argued from that that there must be formation and destruction or removal of collagen But that it goes on continuously in normal tissues is more a matter of inference than knowledge.

Wyckoff: The second major question I should like to bring up for discussion deals with the character of the fine detail seen in 650 Å collagen In our experience, this detail varies greatly depending on the source of the collagen and on other factors that I do not think we are now in a position to define, in this, I differ from others who have attributed its differences to differences in the perfection of the arrangement of the molecules that make up the fibrils.

Let us consider fibrils as distinct from tendon, in which they are imbedded in a great mass of ground substance. Their striations often take on the appearance of discs, such as is seen in Figure 3, and a fibril resembles a series of these discs with a depression between each. Figure 9 shows another fibril of this sort Here the raised striae, repeated at the 650 Å intervals, suggest a system of discs. When one has what I like to call, for lack of a better expression, "cleaner fibrils," no matter what the source of these fibrils may be, bands of threads replace the discs, *it is natural to infer that the discs are such threadlike striae enveloped in a ground substance which has not been removed* Usually, there are two threads to each disc; that is, there are two prominent *threads to each of the 650 Å intervals* They cannot be seen in Figure 9, but they are evident in Figure 10, which is connective tissue from the heart of an adult dog The one disc per 650 Å is replaced by a pair of cross threads, as at B Occasionally, a third thread is visible in the troughs between the pairs; I think it can be seen at C. Sometimes the

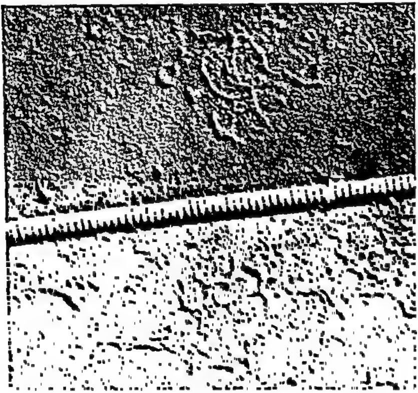


FIGURE 9 A fibril of connective tissue teased from a fragment of rat tail tendon The raised transverse ridges are *ca* 650 Å apart

third thread is not much depressed in relation to the other two, and then the picture merges more or less uninterruptedly into one of continuous 210 Å striations Such an almost continuously striated region can be seen at D Occasionally, one part of a fibril will show markedly two striations on a ridge and one depressed, and farther along on the same fibril a region where the depression no longer is apparent and which looks like a continuously striated 210 Å fiber

In some composite fibers, like the one in Figure 11, these different types of striation may be adjacent and more or less in parallel alignment This reconstituted collagen fiber brings out a point I wanted to make For instance, raised pairs of threads alternating with a depressed singlet can be seen in the region marked J Farther along in the middle (at K), the structure is more disc-like and the thick ridges are not resolved into a pair, this is the sort of thing seen in native tendon

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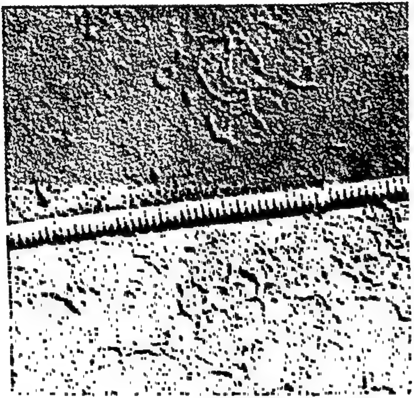

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In some composite fibers, like the one in Figure 11, these different types of striation may be adjacent and more or less in parallel alignment This reconstituted collagen fiber brings out a point I wanted to

solved into a pair, this is the sort of thing seen in native tendon.

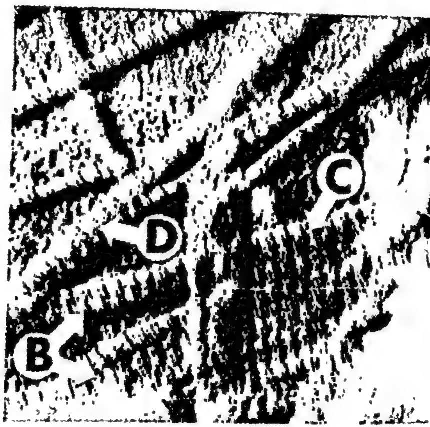
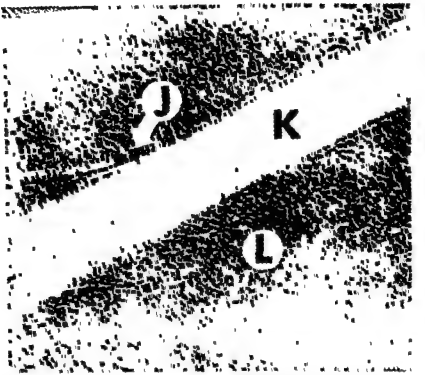


FIGURE 10 *A fragment of tendon from the* [illegible] *dog showing the threadlike appearance of the cross-striae. Each pair of "cross threads" is 650 Å distant from its neighbors Between most pairs a single "cross thread" is more or less evident.* Reprinted, by permission, from Pratt, A W, and Wyckoff, R W. G The fine structure of connective tissue fibrils *Biochim et biophys acta* 5, 166 (1950)

Again, in the region marked L, there are paired elevations but, along the edge of the fiber, places where the third (depressed) band is about equal to the pair will be seen, such an edge is almost continuously striated. *I emphasize these transitions in fine structure because they suggest that the 650 Å and 210 Å fibers are not necessarily totally different kinds of collagen They are extremes between which there can be transitions in fine detail.*

There are not always three threads in each 650 Å interval It is not uncommon to find three, rather than a pair of threads, for each elevation, and more than four substriae have been reported in a 650 Å in-



… collagen in parallel align-
… of the cross striae varies somewhat along the
… ration

terval, especially after phosphotungstic acid staining, as in the early work of Schmitt and his co-workers I do not think we really know yet what this means, and I suspect that a convincing answer will be hard to reach, if only because of the specimen distortion previously mentioned which interferes seriously with the accuracy of measurement

There is still another variant in fine detail which can be seen The striation is almost pyramidal, in other words, one thread will be very prominent, and it will have a somewhat depressed satellite on either side Occasionally, most of the fibers of a preparation will give this type of 650 Å repetition

Porter What is the background material in Figure 11, Dr Wyckoff? Is it unpolymerized?

Wyckoff Yes, not reorganized, dissolved material

Porter Do you suppose that the surface of that material is smooth in the wet state?

Wyckoff: I would imagine so, yes.

Ragan. What do you mean by that?

Porter: Some regions in the fiber are more hydrated than others and actually, in the wet fiber, the surface is smooth. It doesn't show these elevations at all

Wyckoff The only experimental answer I can give to Dr Porter's question is to say that we have tried very hard to prepare frozen, dried fibers, and we have usually failed miserably, as judged by the pictures we have obtained; that is, the fibers have been grossly damaged somewhere in the freezing and drying process. But where we have succeeded, in a few cases, then a fiber is seen in which the striae are scarcely visible Figure 12 is such a frozen, dried fiber.

Porter· This is lyophilized?

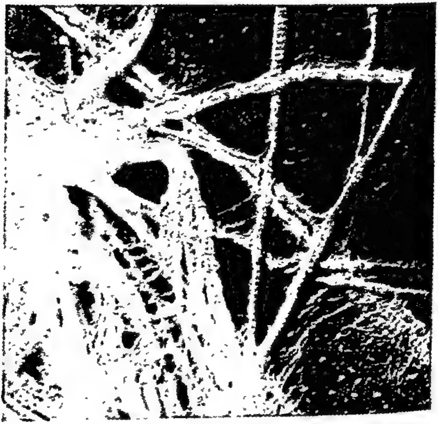


FIGURE 12 A teased fragment of rat tail tendon prepared for electron microscopy by quick-freezing in liquid nitrogen and drying *in vacuo* from this frozen state

Wyckoff. Yes Actually, very few of the striations can be seen A few cross threads are visible, but the depressions are missing

Ragan. Are you implying, then, that the depressions are a result of fixation?

Wyckoff: I think they are the result of dehydration; in other words, I would say that they mark regions where the original fiber was more hydrous.

Porter In sectioned material, again, where the imbedding matrix is left in, the collagen fibers are smooth in outline and the banding is still visible It is not as prominent as it is here in these preparations, but it is definitely evident There are, therefore, differences in density along the fiber

Meyer. Couldn't that be made up by ground substance, or by something which might be called ground substance in a frozen, dried section?

Porter It certainly would fill in between them, yes, in the drying

György Why is the water only in these valleys? Why not in the mountains? Why is the surface smoothed out?

Porter: I said the surface was smooth in a sectioned material where the water has been replaced by another material, by alcohol and finally by plastic

György. Yes, but here it is not fixed, it is lyophilized

Porter. Well, lyophilized, they are dry

György That is what I meant.

Porter: And Dr Wyckoff, I assume, meant to retain the natural form of the fiber by lyophilizing

Ragan: But he took the water out of it

György: That is what I say He took out the water How did it become smooth?

Porter But even in lyophilization, the surface passes through a phase boundary which is what alters the structure

Wyckoff: Yes, I would agree with that.

György Did you not say, Dr Wyckoff, that hydration makes a difference in these ups and downs?

Wyckoff Oh, yes, in lyophilization, one hopes to remove the water without causing the shrinkage which is implicit in the usual drying-down

György The question is why is there shrinkage only in 650 Å?

Wyckoff. All we can now say is that we assume it reflects variations in structure inside the molecule

György There is no answer It changes as more water is added

Porter In some places more than in others

Mirsky But not only at 650 Å The shrinkage can be found at triple intervals.

Meyer: But this 650 Å unit, Dr György, corresponds to something real, as evidenced also in the x-ray diffraction patterns.

Wyckoff: Yes, that is right

Meyer. And the *diffraction is done with fresh, nonfixed material*

Gyorgy. There is no question in my mind about why the water accumulates

Porter: It has to be there in the native fiber.

Wyckoff. Yes It can be imagined that there is a variation in the degree of hydration along the molecule and that this reflects variations in chemical composition along the molecular threads There is further evidence of this in some of the reconstitutions which I shall show in later Figures

Bennett: It is implied that there is a stroma in the fiber from which the water is removed leaving *the form* of the fiber in the lyophilized material

Holbrook Even though you do that, you still leave the skeleton of *the structure showing*, which *is this material in the fiber?*

Porter Nonvolatile.

Wyckoff Right

Holbrook: That represents the morphology of the fiber underneath?

Wyckoff. Yes, that is right.

Bauer But without water.

Wyckoff Basically, I wanted to bring out the variations to be found in fine structure within the 650 Å fiber; thus, we can have the disc-like ridges which are *probably* threads imbedded in a matrix, we can have two threads across each elevation, or three *threads* for each elevation, or we can have more than one of these in a single fiber.

Porter Do you mind if I object to the thread notion?

Wyckoff If you have a better word than "thread" for what we see

Porter: "Thread" implies a structure which goes across the fiber, as though wrapping around it Actually, these are elevations in a longitudinal arrangement, variations in amount of material in the longitudinal direction, rather than morphological threads passing around at *the point* of the *striation*

Wyckoff. I wouldn't *know* whether or not there was a *real* thread there

Bauer: I do not see why threads are necessary.

Wyckoff. I did not imply they were necessary, but *one* must have words to describe what is seen.

Travell Does the term "*thread*" *imply a core through the* center, that it is wrapping around something?

Wyckoff: No, not at all It *merely implies that* what *is seen* in the pictures *is* something that looks like a thread across the fiber.

Dempsey. We tend to think of these substances as long molecules, of course, and therefore we expect or hope that we will see something that has a longitudinal or a transverse orientation. Is there any suggestion that this periodicity might represent a cross-linking of longitudinally oriented elements?

Wyckoff. I do not know what it represents. The point I should like to make is that there can be variations in the fine detail of the fibers, that is, there can be two of these threads, whiskers, whatever you want to call them, or three of them. Another point is that I do *not* know if there is any connection between this multiplicity and the source of the material; it would be *important* to know whether or not there was such a connection. The data that bear on this point are not known.

In dealing with problems raised by the variety of this fine detail, care must be taken not to be misled by beat-like superperiods which are sometimes seen in collagen fibrils. Figure 13 is one example of such

FIGURE 13. A collagen fibril showing ridges that repeat themselves at an interval of more than 650 A.

beats, which I show because they can be troublesome when dealing with material that is not very clean In Figure 13, there are definite ridges far more than 650 Å apart. In this particular case, they are quite oblique to the axis of the thread but they do not need to be. I do not know if they are ever exactly normal, but they can be nearly so They are not confusing if the fibers are clean enough to show the underlying 650 Å striae, nor are they confusing if the striae can be accurately measured

Lillie. What is the cleaning process?

Wyckoff. Usually nature does it. On some fibers not as much ground substance adheres as on others. These are not chemically cleaned The superperiods, if that is the best term, commonly arise from a slight twisting of one fibrillar unit about another. Sometimes this can be seen and sometimes not

That is all I want to say about fine detail within collagen fibers To sum up, the detail is primarily interesting in two directions It has a great bearing on our understanding of the molecular structure of the fibrils, and it would be of further importance if any connection between these fine structures and the source of the collagen fibers could be discovered.

If there is no further discussion on this point, I shall move on to my third question, introducing it by saying that nearly all the information discussed thus far has been gained from fibrils which have been dried on a substrate and from samples which have been obtained by teasing apart bits of tissue The collagen fibers prepared in this way, as the previous Figures have shown, appear as more or less flattened cylinders I want to introduce now the question of what is seen in sections through connective tissue.

Connective tissue, of course, has to be studied *in situ* if we are to know what it is like in any but such massive aggregates as tendon In addition, the very differently prepared samples that are needed for sectioning can reasonably be expected to throw light on the relation between these flattened fibrils and the fibrils, even of tendon, as they naturally occur.

Before going on with this, I should like to say that massive collagen, such as tendon, is extremely difficult to thin-section for electron microscopy. It is probably the toughest material that we have ever tried to section, and, even when adequately thin-sectioned, it is very difficult to photograph because the preparations vary greatly in density or electron opacity at different points As a consequence, the samples very easily rupture or distort, and the tendon is likely to move in the electron microscope while being photographed This is said by way of apology for the quality of the electron micrographs of sectioned tendon which

follow We have often found it necessary to take what information we have been fortunate enough to get even though the photographs are not always as nice as we would like The following series of Figures, then, are an indication of what we have seen when sectioning tendon The tendon shown is from two sources, one from the wing of a chicken, the other from a rat tail

Figure 14 is from chicken wing The section has been cut more or less along the direction of the fibers of the tendon The tendon was fixed in what we commonly call "neutral osmic," which is actually osmium tetroxide added to a buffer of neutral pH Even at this low magnification, the 650 Å striations are visible It is characteristic of our sections that the collagen fibers are usually separated from one

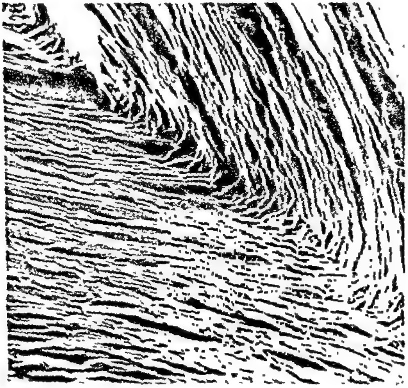


FIGURE 14 A thin section through a tendon from chicken wing The slice is along the axis of the tendon and shows the collagen fibers grouped into small bundles Printed, by permission, from Noda, H and Wyckoff, R W G *Biochimica et biophysica acta* (In press)

another. Undoubtedly, the methods of preparation have removed most, if not all, the ground substance so that the separation is due in part to this abstraction of some of the tendon and in part to a shrinkage of the fibrils themselves.

The higher magnification of Figure 15 brings out very clearly the 650 Å striations, as well as the fact that, when the fibers are cut on the bias, they have an appearance very strongly suggestive of tubes rather than cylinders That is something which surprised me very much but which has been seen repeatedly.

Figure 16 is a section parallel to the axis of the fibers. It shows their striated character, as well as a superperiod on the 650 Å repetition

FIGURE 15 A section such as that of Figure 14, photographed at a ... the individual collagen ... e. *Many of the fibers in* ... hat on the bias, appear hollow. Printed, by permission, ... oda, H., and Wyckoff, R. W G *Biochimica et biophysica acta* (In press).

FIGURE 16 [illegible]

[illegible] spacing

It can be seen, especially in several fibers at the bottom of the picture, that every third 650 Å striation is weak, and this gives rise to a dominant repetition every 1950 Å. It will also be noted that some of the fibrils are themselves sectioned or torn open, although continuing to manifest their striations.

Figure 17, at low magnification, is a tendon almost transverse to the direction of the fibers, and it is easy to see the bands into which the individual fibrils are grouped.

Porter: Do they show up as tubules there?

Wyckoff: They will at a higher magnification. Figure 18 illustrates it clearly. The striations show through the cut ends, and there is created the impression that we are looking at tubes cut on the bias to show the striations on their back surfaces.

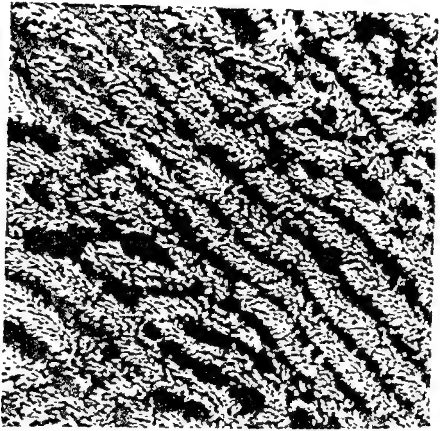


FIGURE 17 A section of chicken tendon cut almost transverse to the tendon axis The grouping of the fibers into bundles is more apparent than it was in Figure 14.

Bauer If they were cut on a slant, they would exhibit this same appearance, would they not?

Wyckoff: You can tell whether you are looking at the front or back side of the fiber.

Porter: There should be a pronounced difference in density between the shadow of the whole fiber and the half fiber, which is certainly true in Figure 18 But, of course, it could be a cylinder and still show that

Bauer: I think it should be cut on a slant

Porter I might point out, Dr. Wyckoff, that the imbedding matrix has been removed here and that anything that goes with it in the removal has been taken away from between the fibers

Wyckoff: Much has been taken away before the imbedding substance

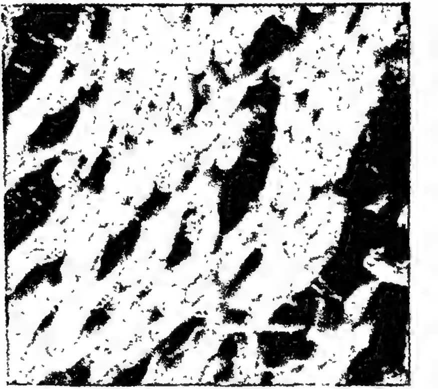


FIGURE 18 Part of a section of tendon such as in Figure 17, photographed at higher magnification The striations are visible not only along the surface but through the cut ends of the separate fibers. Printed, by permission, from Noda, H, and Wyckoff, R W G *Biochimica et biophysica acta* (In press)

ever got in I don t imply for a moment that in the tendon the fibrils are tubes

Porter I would not be surprised

Wyckoff I don't imply it, but I think that, if they appear here as tubes, one has to wonder very much as to what was inside them when they were in the intact tendon We have now quite a few pictures which certainly give the impression of tubes

Dempsey How much do they change in diameter as they dry down upon the film?

Wyckoff How much have they shrunk?

Dempsey No If they are either cylinders or tubes, if they are originally circular in cross-section, then, as they are put on the film and

dried down, they should flatten to some degree.

Wyckoff: These are sections

Dempsey: Yes, they are sections, but I am thinking of the spreads of native tendon now. Is there any information about how much they flatten as they dry down?

Wyckoff. There is no accurate information. I think it is safe to say, from the replica work that has been done, that they are not uniform in diameter.

Porter In tendon, certainly not, but in some of the other tissues they are.

Wyckoff. In some tissues they are surprisingly uniform, yes.

In common with many other people, we have been interested in look-

FIGURE 19 A section, cut approximately parallel to the fiber axis, of tail tendon that had been swelled in glycerine Printed, by permission, from Noda, H, and Wyckoff, R W. G *Biochimica et biophysica acta* (In press)

ing at tendon which has been swelled, and the next group of Figures deals with swelling In my book (1), I showed some acid-swelled tendons which had been dried down, they exhibited not only the extreme [illegible]

tendon, and Figure 19, for instance, is tendon which has swelled after treatment with glycerin There is a shredding of its fibrils, following their rupture The rupture and shredding going on in fibrils that still retain some striations can be seen Such fibrils in various stages of disintegration are better shown in Figure 20 At the center of this photograph are fibrils that are tube-like. And there are one or two places on this photograph where there seems to be a considerable periodicity in the tiny fragments, possibly in the neighborhood of B

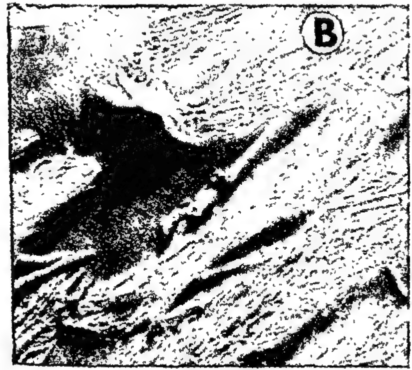


FIGURE 20 A section, similar to that of Figure 19, through swelled tendon photographed at higher magnification

Porter: Did you expect the glycerin to penetrate into certain parts of the fiber?

Wyckoff: Yes.

Meyer: This is concentrated glycerin?

Wyckoff: We started out with 50 per cent and then went to 100

Meyer: You don't wash the glycerin out afterward?

Wyckoff. We did, yes We went through alcohols, actually. I started out with a totally different and utterly erroneous idea as to what I was going to achieve, but we did get very nicely swelled and frayed fibrils. This treatment exposes much better than does acid-swelling the longitudinal filaments that are a component of the fibers.

Other

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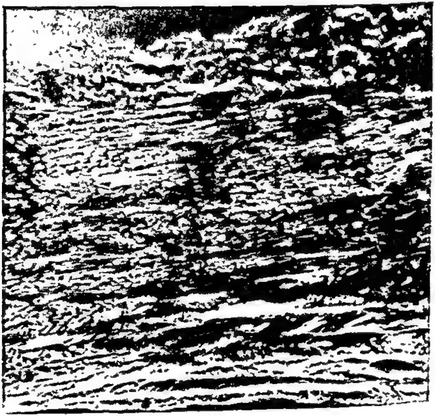


FIGURE 21 Connective tissue sheath of rat sciatic nerve The central band consists of striated collagen.

mesoderm of chick embryo is an example. In the many other photographs we have made of similar material, not only are the 650 Å striations seen but also, in some places, what seem to be the 210 Å continuous striations; in other words, both types of fibril are present

The next group of Figures is drawn from our work with nerve, and I use them merely as another example of connective tissue in section In Figure 21, one sees at low magnification the peripheral covering of a sciatic nerve of a rat. All the nerve fibers are in a region below the bottom of the picture In Figure 22 at a somewhat greater enlargement, the individual fibrils are clearly visible and in places their 650 Å striations are just discernible. The Figure is, however, most interesting for the directions of the fibrillar bundles making up the perineural band Most of the fibrils run diagonally upwards from the bottom left, but, in so doing, they enclose another differently directed bundle coming out as

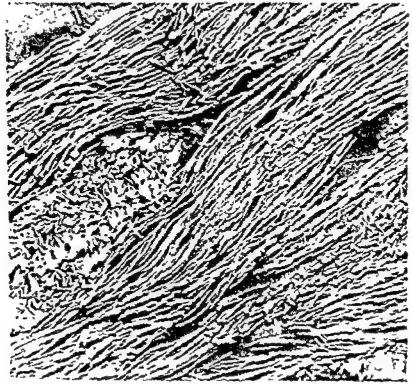


FIGURE 22 A portion of another region of the collagen band of Figure 21, photographed at a somewhat higher magnification

an island in the middle left of the picture which is being cut almost transversely.

Porter: Wouldn't it be interesting to point out the uniformity of those fibers, Dr. Wyckoff?

Wyckoff: The widths are very uniform, obviously, and they are all 650 Å collagen, as far as we have observed. We haven't seen any 210 Å fibrils in this particular group of sections.

Holbrook: Is this the sheath of the nerve?

Wyckoff: Figure 21 covered a section through the entire perineurium Connective tissue fibrils are also seen between the individual nerve fibers in the body of the nerve, but I do not have any pictures of those Figure 23 is of a few of the previous fibrils at higher magnification This collagen was, in our terms, very dirty. In a few places, the 650 Å striations appear rather well, but in most of the fibrils it is obscured by enveloping material.

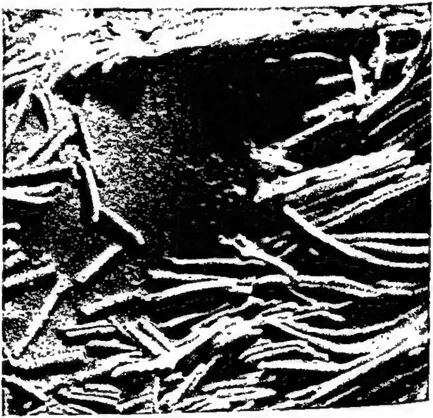


FIGURE 23 Collagen fibers at still higher magnification than previous Figures A few are "clean" enough to show their 650 Å striations

This is all I have to say about what is seen in connective tissue that has been sectioned and about its relation to the apparent structure of dried collagen This might be a good place to stop for discussion

Dempsey Dr Fremont-Smith believes these conferences ought to be exercises in communication between people with different training, and I should like to get this translated, if possible, into the conventional terms that a histologist uses. This perineurium that we are looking at, to a histologist, should have reticular fibers and some coll[illegible] s The collagenous fiber is an enlarge[illegible] e collagenous f[illegible]

[illegible] ...ity of the fibrils or fibers s[illegible] ...ed if they come under the category of what the histologist regards as the reticular fi[illegible] ...wer order of magnitude

W[illegible] ...optical microscope

De[illegible] ...dual fiber which we see, then, is a bundle of these unit fibrils?

Wyckoff What you see would have to be a bundle of what you saw here

Dempsey. The other thing that, in histologic terms, seemed striking to me, was the ease with which what we always think of as very dense collagenous fibers may be separated into their component parts They are separated by the process here of sectioning, that is, they are made resolvable by the process of sectioning They are also separated easily by immersion in glycerin That latter experiment interested me very much It has been repeatedly suggested that the difference between form and intrinsic birefringence can be determined by observing birefringence of objects that are immersed in a series of media of different refractive indices, such as glycerin and water mixtures If the ultrastructure is destroyed to such a degree and the individual elements become amassed into a net-like organization such as these pictures indicate, this immersion procedure seems to be a rather hazardous technique Immersing fibers in the glycerin-water mixture causes a separation of the individual components so that their orientation is changed

Wyckoff. I do not think the general direction of the orientation has changed for what is left of the fibers

Dempsey Yes, but some of the big fibers seem to have frayed out like the end of a rope, and they are not so oriented as when the rope is twisted into strands in its original form

Wyckoff. That is certainly true There is surely some fraying

Dempsey Then, any change in observable birefringence might be due to the change in orientation of the micelles rather than to the dif-

ference in refractive index between the fiber and the immersion medium

Wyckoff: Very easily.

Meyer: But it is not certain that a lot of stuff is not taken out by using the glycerin-water mixture. That is still the interpretation of the birefringence data and it may be correct because, before you could draw such a conclusion, you would have to prove that the fluid which you extract is optically empty

Wyckoff: Yes, that's right.

Porter: Chemically empty.

Meyer. Yes, chemically or optically empty, that is, that there are no substances which have a definite refractive index.

Wyckoff: I think it unlikely that nothing is removed. You are, in a sense, opening up the fiber, just as with acid, and by the opening-up process, you are undoubtedly removing things.

Robb-Smith. Have you, or has anyone, studied the Plenck-Laidlow (10) sheath, or whatever one likes to call it, the sheath that is argentophil and lies between the myelin and the neurilemma? It might be interesting, as it is generally thought to be of neurectodermal origin rather than of fibroblast origin.

Wyckoff: No, we have not.

Robb-Smith: It would be nice to know whether it has the same periodicity as the mesoderm type of fibril.

Meyer: I noticed that you did not want to commit yourself as to whether these are tubes or cylinders, Dr. Wyckoff. Do you believe that the individual fibers which make up a cylinder or tube are separated by some material or are simply in juxtaposition to each other? You recall that Schmitt and Gross stressed the point that the cross striation in the collagen bundles always coincided, whatever their periodicity was

Porter: In phase

Meyer. That they are all in phase, yes, that all the striations are in phase.

Wyckoff: Across the bundle?

Meyer. The one picture certainly looked like tubes. Would you think that that is a more likely structure of the collagen fiber?

Wyckoff: I wouldn't want to go beyond saying that the fibrils look surprisingly like tubes in a number of pictures.

Meyer: Would you think that this is a more likely picture of the collagen fiber?

Wyckoff: No, I wouldn't think so

Meyer: Is it conceivable that any artifact may be responsible for blowing them up into tubes like a deflated sausage casing?

Wyckoff: No, I don't think so

Fischel: Is the fact that there is no discernible change in density on the rim of the cross section more in favor of a cylinder concept?

Wyckoff: There is a variation in density That is what interested me.

Fischel: There is variation from top to bottom but not consistently around the periphery, is there?

Wyckoff: What bothers me more than anything else is the straightness of the cross-striae, as seen across the cut

Robb-Smith: The heavily shadowed ones did look like tubes.

Wyckoff: I am not trying to sell the idea that these things are tubes because I am not completely sold myself. I am surprised how tube-like the fibrils look, particularly in these recent observations on sectioned [illegible] But draw-

[illegible] spiral arrangement?

Wyckoff: No, I don't think so

Porter: Somebody else proposed that it was a tube, but we have found out since that it is not.

Dempsey: The myofilament may still be a tube

Porter: That is getting down pretty fine I don't think you want to resolve that

Lillie: Dr. Wyckoff, is staining of collagen fibers with anilin dyes likely to make any appreciable difference in their electron micrographs?

Wyckoff: I think it unlikely, but I don't know

Dempsey: Certain things are used to enhance the contrast in the electron microscope osmic acid and phosphotungstic acid and so on. Is there any information on the electron density of a series of metals? Can it be stated which things give the greatest electron density and which give lesser?

Wyckoff: Yes It is an affair of atomic weight and density

Dempsey: It is purely on an atomic-weight basis?

Wyckoff: Yes, it is on that basis, so the dye which could be loaded with a heavy atom is the one you would be interested in trying.

Porter: Mercurochrome.

Dempsey: Yes.

Wyckoff: Or impregnation stains, though that is another problem Where you are dealing with an impregnation and not a stain, you can quite surely localize the effect

Dempsey: Yes, but that is a different matter because the metal builds up on itself in almost a photographic way

Wyckoff: It can be said with considerable assurance that observable results can be expected with the electron microscope where dealing

with an impregnation; whether that is so with a dye is a much more questionable thing

Dempsey. If a dye were tagged with a heavy metal, then it should become visible in the electron microscope.

Wyckoff: If enough of the dye is taken up by the tissue.

Lillie: Mercurochrome is essentially a mercury-loaded eosin and it might work.

Potter: But it isn't taken up differentially; it stains everything, in our experience

Dempsey. This is a good chemical problem

Lillie. You ought to be able to start to work on that. The amount of eosin that is taken up can be limited by regulating the pH of the solution and making it much more specific in some tissue elements than others. You can even balance it with basic anilin dye in solution and get the same sort of selective take-up of the red dye as we do by balancing eosin azure for tissue staining, at specified pH levels If [illegible] hould be limited [illegible] d deal more information with regard to what we see in our stains and our electron microscope

Lillie We are trying to put two things together

Wyckoff. There is a very large field of investigation open to somebody who has the facilities.

Holbrook: Has there been any work done on that that you know of, Dr Porter?

Potter No Phosphotungstic acid has been explored more than anything else, perhaps It is taken up, preferentially, by certain parts of the collagen fiber, and some of the intraperiod striations may result from the absorption of phosphotungstic at molecular margins rather than elsewhere in the molecule I suspect that the phosphotungstic acid describes partitions in the structure of the fiber. Besides this sort of staining, one could conceivably devise substrates with a heavy atom in them, which atom might then be deposited at the site of the enzyme action

Holbrook: That would be very helpful.

Wyckoff. The next major question I want to bring up deals with the basic problem of how collagen fibrils are formed The aspect of this I should like to discuss deals with reconstituted collagen Work with reconstituted collagen, as far as I know, had its beginning in the observations made by Nageotte (11) about twenty years ago in which he showed that, from solutions of suitable acid-dissolved collagen, a fibrous material could be reprecipitated that, by the tests then avail-

able, seemed much like the original collagen. Following Nageotte's work, Corey and I (12) became very much interested in whether or not we could get x-ray diffraction from this reprecipitated material and in this way determine if it really was collagen. This was, in fact, the origin of my interest in problems of collagen. We did obtain quite good collagen patterns from the reprecipitated fibers, and to us it seemed good evidence that they remained substantially like the original tendon.

Along with many other people, including Dr. Porter, I have been using the electron microscope in late years to see what these reprecipitated fibers are like; it is certain that they can have a periodic fine structure which is indistinguishable from that of native collagen. It is most important that both the 210 Å and the 650 Å collagen fibrils can be precipitated from the same solution under suitable physicochemical conditions. I have mentioned it before and would like to emphasize it again.

The work which we have been doing in this field, more or less as a side issue to our other problems, has been directed primarily toward finding out the principal physicochemical factors which influence the fine structure of precipitated collagen. I do not want to give a resume of our work, but I would like to mention a few results that are pertinent to this basic question of how collagen is formed.

The source of the material in the following Figures has been rat tail, Achilles, and finger tendon. For our purposes, we have seen no difference among these tendons, nor have we seen any difference between the solutions of tendon from young and very old rats; we have done no work with newborn rats. All of these tendons swell, and they partly dissolve in very dilute organic acids. We have routinely used a 1 in 10,000 dilution of acetic acid or a 1 in 100,000 dilution of formic acid as the dissolving media. Acids like citric and very dilute hydrochloric can be used and result in useful collagen solutions. The particular acid used for solution does not seem to influence the fine structure of the precipitate obtained later. The texture of the precipitates formed by salting-out from such a solution, with sodium chloride, sodium sulfate, or ammonium sulfate, for instance, is coarser for the less concentrated precipitants. This has been a general finding. The fibrils that we have obtained in this fashion have usually been without a periodic fine structure.

Figure 24 is an example of such a precipitate. This is an acetic acid solution which has been salted out with a tenth molar sodium chloride solution, and periodic fine detail is missing.

Bauer: At no place in the preparation is the period seen?

Wyckoff: I wouldn't go so far as that, but an overwhelmingly large amount of the precipitate consists of such ropes.

FIGURE 24 Nonstriated collagen obtained by the addition of sodium chloride to an acetic acid solution of tendon Reprinted, by permission, from Noda, H, and Wyckoff, R W G The electron microscopy of reprecipitated collagen *Biochim et biophys. acta* 7, 494 (1951).

Porter: Did you give it plenty of time? Time is a factor in our experience

Wyckoff: Yes, time is a factor, and it is true that the nicest 210 Å striated fibrils we have seen have been obtained by precipitation with sodium chloride, either by itself or, as in Figure 25, in admixture with a pH adjusted sodium acetate solution. We have used a sodium acetate solution here because, by itself, it did not produce a precipitate As you can see from Figure 25, this precipitate consists of very nicely striated 210 Å fibrils.

FIGURE 25 Closely striated (250 A) collagen precipitated from solution by the addition of buffered sodium chloride

Holbrook That is a precipitated collagen?

Wyckoff: This is collagen precipitated from the solution

Bennett Do you ever find the tube-like structure in the reprecipitated material?

Wyckoff I haven't Actually, we have only had the tubes very recently, and in sections

Bauer Tube-like

Porter This was dilute sodium chloride?

Wyckoff In this particular case, it was 0 2 molar So far, our results in getting striated fibrils through the use of sodium chloride, with or without buffer, have not been reproducible

Bauer Was that true irrespective of concentration?

Wyckoff The vast majority of our sodium chloride precipitated ones

have been structureless, so that I do not think we have found the crucial factor.

Porter: You certainly have to duplicate every condition imaginable to get the same thing.

Wyckoff. If a buffer is used as a precipitant, then the results are reproducible; practically all of our work has been devoted to seeing what we get by using buffers of various ionic strengths and various pH's, as well as the result of using different kinds of buffers as precipitates

Bauer· How about temperature?

Wyckoff. We have done very little in studying the effects of temperature When we bring about precipitation with buffers, in our experience the striated fibrils are usually of the 650 Å variety We have found the valence of the buffer anion and, apparently, its specific nature important Thus, the addition of sodium acetate solutions of adjusted pH, as an example of a univalent salt, has failed to give the same kind

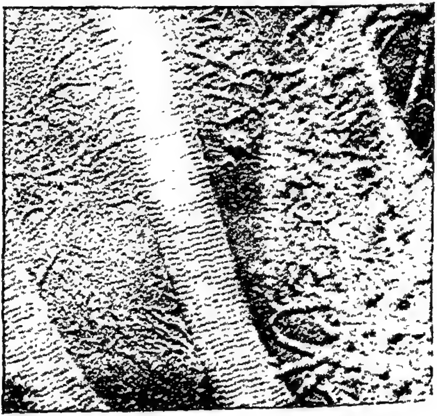
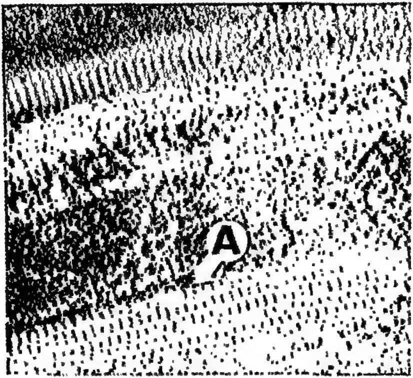


FIGURE 26 Collagen precipitated by the addition of pH 4 6 citrate buffer



...constituted collagen precipitated with ... buffer

of precipitates at corresponding molarities as we obtain by using oxalates or citrates In this sense, and in this sense only, the phenomenon has been ion-dependent

In Figures 26 and 27, the material has been precipitated with citrate buffers, but very similar results would follow the use of oxalate solutions of controlled pH Fibrils with the best developed striae have been obtained at molar salt concentrations which have varied from about 02 molar to about 2 molar And, as I said before, the pH is of the greatest importance

Figures 26 and 27 illustrate what we see at various pH s, with 03 molar citrate buffer In Figure 26, one observes at the right the kind of small fibers which precipitate below a pH of about 4 6 Beginning at about pH 4 6, nicely reconstituted collagen, like the central fibril running vertically, appears Between about 4 6 and a little over 5 0, the precipitate is predominantly such 650 Å striated material Figure 27 is

another example of 650 Å fibrils, which is of interest in showing what seems to be the development of striae from the mass of unorganized molecular particles in the background. The transverse fine structure can be seen gradually blending into the background in various places One striking consequence of this is the irregularity in outline of the fibers In other words, the edges of the fibers are extremely ragged Not just cylinders and tactoids are found.

Porter. How would you describe the wet fiber before it is put on the grid?

Wyckoff I shall do a lot more work first!

Porter: In this case, that lower part of Figure 27, where the macroperiodicity comes out, is obviously the center of the fiber.

Wyckoff. The macroperiodicity is also on the margins, but the ridges are very much depressed.

Porter: Do you suppose it is more hydrated at the margin in the wet fiber, where the collagen is adding on all the time?

Wyckoff· I think it is, yes I think we are seeing an actual organization going on along the edges of these fibrils. Otherwise, I do not know how to explain the islands of order reaching into the disorganized background

Lillie. Are these fibers generally deposited in the parallel manner as in Figure 27?

Wyckoff. That is purely an accident. They are found in all sorts of order and disorder, often snarled up

Porter. Where the macroperiod is not seen, some people would contend that it is only because the differences between the 210 striations are not sufficiently pronounced

Wyckoff. I don't quite understand what you mean.

Porter: At the margin, you point out the uniformity of striations at 210 It could reasonably be proposed that they appear uniform because the differences between them are not striking enough to be resolved by the microscope. It is only when the differences are superimposed, as in the center of the fiber, that you begin to see the macroperiodicity

Wyckoff· But at A, for instance, it is very thin and yet the 650 Å macroperiod is quite clear.

Porter: Yes, that is true.

Wyckoff· Whereas in other places there is a quite deep, almost uniform layer

Porter: But if there were slight differences and they piled up, as they do toward the center of the fiber, then there would be an exaggeration of the 650

Wyckoff: Yes.

Fischel· Have you ever been fortunate enough to focus on the longi-

tudinal end of the fiber to see if the ragged effect and the periodicity extend out into the amorphous substance at the tip?

Wyckoff: You can see it in a way in Figure 27, as the central tactoid tapers off into the background

Bauer. We can see it in the frayed ones.

Wyckoff: In the frayed ones, you can definitely see it

Bauer. And all through in Figure 27, it can be seen at the end

Wyckoff. But the order disappears progressively and it cannot really be said at what point the fiber has stopped, at least, I wouldn't be quite sure where it ended

Fischel Then the periodicity stops longitudinally long before it does in the perpendicular plane, that is, it extends out into the medium on each side rather than at the tip

Wyckoff: You would have a harder time seeing it out there

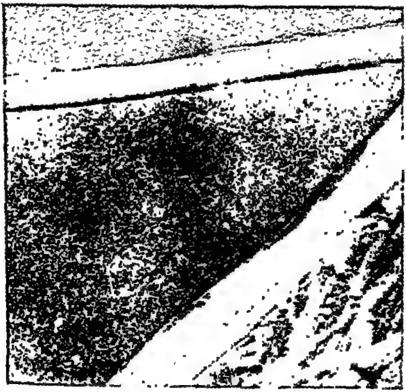


FIGURE 28 Collagen fibrils precipitated with pH *ca* 5 2 buffer The striations, though perceptible, are much shallower than in preceding photographs

If, to return to our series, the alkalinity is increased a little, the striae gradually become less striking. As is seen in Figure 28, for which precipitation took place a little above pH 5, the fiber at the top is almost smooth. If the pH is raised a little more, to somewhere around 5.5 depending on conditions, a mass of small, unstriated fibrils again are produced. As Figure 29 shows, they are suggestive of what was produced on salting out and by precipitation on the acid side.

One can, by studying pictures such as these, see something of how the fibrils form. Developing order can be traced back into the nonfibrillar background. Figure 30 is not a dissolving fiber, as you might imagine. What is especially interesting is the presence of definite striations in the rather unformed mass of molecules among what look more like tree roots than anything else.

Travell. How long does it take for a fiber like that to form?

FIGURE 29. Collagen precipitated with less acid (pH *ca* 5.5) buffer.

FIGURE 30. Precipitated collagen showing regions of partly formed fibrils in which the 650 A striae are already visible. Reprinted, by permission, from Wyckoff, R. W. G.: The macromolecular structure of biological materials. *Science in Progress*. Baitsell, G. A., Editor. New Haven, Yale University Press, 1951 (p. 203).

Wyckoff: It is sufficiently instantaneous so that we have not had much luck in trying to determine it. But you do see the organizing process going on.

Porter: The striae form an arc, don't they, and they tend to be in phase?

Wyckoff: They do tend to be in phase throughout the whole mass.

Holbrook: Would you assume, Dr. Wyckoff, that there will be, in the next second or at some time, a fiber which you can see at that point? (Figure 30)

Wyckoff: I don't know.

Holbrook: If you could click it by seconds?

Wyckoff: I think not, in the sense that, for these preparations, the

If, to return to our series, the alkalinity is increased a little, the striae gradually become less striking. As is seen in Figure 28, for which precipitation took place a little above pH 5, the fiber at the top is almost smooth. If the pH is raised a little more, to somewhere around 5.5 depending on conditions, a mass of small, unstriated fibrils again are produced. As Figure 29 shows, they are suggestive of what was produced on salting out and by precipitation on the acid side.

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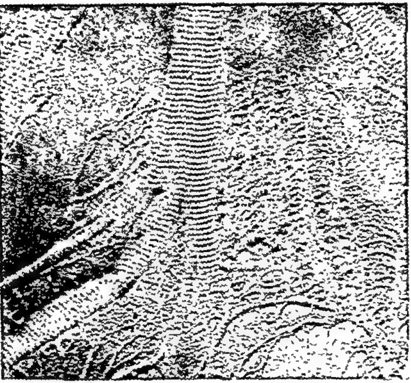

FIGURE 30 Precipitated collagen showing regions of partly formed fibrils in which the 650 A striae are already visible Reprinted, by permission, from Wyckoff, R W G The macromolecular structure of biological materials *Science in Progress* Baitsell, G A, Editor New Haven, Yale University Press, 1951 (p 203)

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Travell· How long does it take for a fiber like that to form?

FIGURE 29 Collagen precipitated with less acid (pH *ca* 5.5) buffer

We have tried, for instance, salting out three or four times and then precipitating with buffer.

Bauer: What did you get?

Wyckoff: We still got striated fibers But it has been necessary to carry out the re-solutions with acid A seeming solution can be obtained with distilled water, but this is not a true solution and fibers are not precipitated from it.

Figure 31 is interesting for the extravagant thinness of some of its fibrils *Most of those in the bottom half of the picture are so thin that* the irregularities in the background can be seen through them It seems

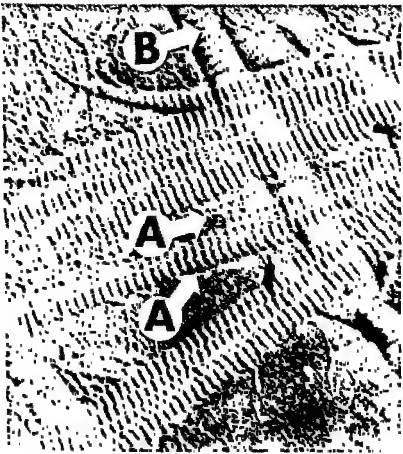

FIGURE 32 Still another photograph of reprecipitated collagen showing the fine structure of the 650 Å striations

fibers have formed pretty fast. This is, I think, followed by a slow further development It is somewhat analogous to crystallization.

Bauer. Can you redissolve and reconstitute these fibers indefinitely?

Wyckoff: We have been trying to do that lately

Bauer: How far have you carried it?

Wyckoff: We have not carried it beyond three or four reprecipitations Usually there has been a certain residuum which has not dissolved again, and we have been stopped by this loss of material. To go further means that we would have to start again with much more tendon We started this experiment with the notion of seeing whether we would fractionate out something necessary for the formation of striae.

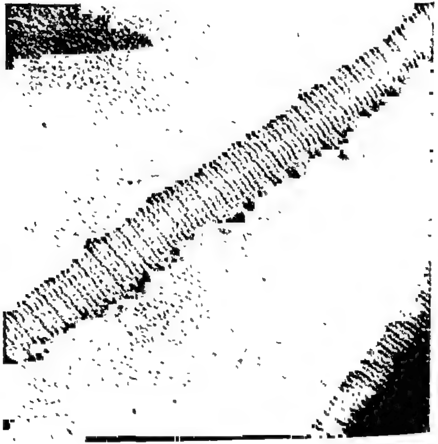

FIGURE 31. Another photograph of developing striated collagen fibrils. Those forming in the bottom half from the matte of molecular threads covering the entire substrate are very thin

have not tried to determine their thickness It is my feeling that shadowing as a method of determining thickness offers more hope than results

Bauer You have a rough approximation, at best

Porter: Dr Ragan said something about scar formation being relatively acellular Is it acellular from the beginning of the wound healing process, or is there a good population of fibroblasts in there initially?

Ragan. Initially, it is certainly not acellular

Porter That could be held responsible for much of the collagen

Ragan I am curious about turnover of collagen, that is, after the scar tissue has been laid down.

Bauer. We don't know enough about turnover

Ragan We do not All measurements are indirect, and if there is any defect in something that is as simple as serum protein, the defects are immeasurably multiplied in carcass protein turnover The people who are working on isotopes now are somewhat concerned about the data relating to serum protein turnover

Dempsey There is a good deal of variation in turnover depending upon the location of the collagen Ten years ago, we were studying the effect of thiouracil on the thyroid gland As is well known, one can make a rat's thyroid grow from an organ that weighs about 8 mg to one that weighs 50 or 70 mg by this antithyroid drug Histologically, it not only increases its parenchyma but also the stroma Then the animal which has grown this greatly enlarged organ, with greatly increased stroma, can be taken off the thiouracil Within a week, the enlarged gland melts away and goes back to normal appearance Here then is a situation in which an organ first lays down a great deal of collagen and then destroys it And, contrariwise, there are other situations in which the collagen, once formed, remains for very long periods of time

Robb-Smith Of course, one has another example of this building up and laying down with the Lipschutz type of experiment in which fibromata are induced in spayed guinea pigs treated with estrogens, but when there is competition with testosterone, there is rapid regression of the "tumors" We have been doing some experiments, keeping the guinea pigs on a scorbutic diet, and find that they still form "tumors" even though they die of scurvy, but they die quicker

Dempsey Jack Wolfe showed some years ago that there was growth and regression of the argyrophilic reticular tissue in the uterus of the rat during the reproductive cycle

Meyer Aren't we getting a little off the subject? There are really two questions here, the turnover as opposed to organization and reor-

to me at least unlikely that the thin fibril in the lower right corner is a flattened tube, or cylinder either. But in spite of its thinness, order is apparent throughout

In Figure 32, there are many regions where one sees very clearly three threads, or whatever you wish to call them, instead of the more usual pair, to each 650 Å elevation There is also visible, in the region above the fiber marked A, a very considerable particle order, reaching out beyond this fiber It is striking. I should also like to draw attention to the fine molecular threads that are running almost longitudinally along the back of the large fiber marked B

Bennett. In its arrangement around the constituted fibers, some of this material is reminiscent of the material between fibrils which Dr Porter showed last year.

Porter. Yes, except that this is more closely striated than that It looks very much as though the forming fiber or crystal, if you like, were exerting some influence beyond its margins.

Bauer It is still going on

Wyckoff Yes, it is going on. It is growing out.

Porter. It is not as compactly organized at the periphery as it is at the center

Wyckoff. My interpretation is that the ordering process, whatever it may be, is seen best on the periphery.

Meyer. Is there any idea of how deep these fibers are? If you had a cross section there, how high does this opposition take place?

Wyckoff We haven't tried sectioning because of the amount of material it takes, but we have tried by freeze-drying to do the same sort of thing, and we have had no luck.

Bauer: Could the thickness be determined by shadow testing? You are interested in thickness, are you not, Dr. Meyer?

Meyer. Yes

Wyckoff: You are worried about the thickness before it dries down?

Meyer. No, the question is why they grow. This looks as if they grow only in two dimensions. If the bundles would grow in three di- [illegible] crystallization was assumed, the [illegible] e to align these macromolecules in phase Why should that go on only in two dimensions and not in three?

Porter. It proceeds all around the face of the crystal.

Meyer. It looks very thin to me, for example, where the one fiber overlies the other, unless you assume that these areas align the underlying cross-striation this way, and the other the other way.

Wyckoff The fibril is extravagantly thin in a region like A, for instance. It can't be many of those molecular threads thick, But we

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Meyer Aren't we getting a little off the subject? There are really two questions here, the turnover as opposed to organization and reor

ganization. The question to which we would all like to have an answer, and which Dr. Holbrook tried to get Dr Porter to tell us is, how does what Dr. Wyckoff told us apply to the formation of collagen in nature?

Porter: Perhaps we might go back even further into the nature of things and ask Dr Wyckoff to tell us what he thinks is the shape and form of the collagen molecule, the smallest unit that can still be called collagen.

Dempsey: That is known as passing the buck!

Porter Is it a 650 Ångstrom Unit, 650 Ångstroms long, with three bumps on it, or is it a 210 Ångstrom spheroidal particle, or what is it?

Wyckoff· I do not know I have always thought of it as being a polymer thread with at least two periodicities, at 210 Å and 650 Å.

Porter: You mean, then, that it is a unit 650 Ångstroms long, with variations in density along its length?

Wyckoff: I thought of it more from the standpoint of variations in reactivity along the length.

Porter One cannot help but be impressed by the wavelike character of these macroperiodicities It is tempting to say that this is a reflection of a dynamic state, that the collagen fiber is—

Wyckoff· Oh, I thought you meant the molecule.

Porter: This wavelike form represents a migration of density, or, let us say, a wave of condensation

Wyckoff· Yes, but any repetitiveness of that sort can be either within the molecule or in the arrangement of the molecules

Porter And it cannot result in any transposition of material forming the fiber It can only be like a wave of compression in a spring. Do you think it is conceivable that the macroperiodicity, at least, could be a wave of compression or condensation which moves along the fiber?

Wyckoff· It is a new idea to me to think of it in terms like that.

Meyer: The question which Dr Porter asked goes into the dimensions of molecular structure These are not resolvable at the present time with the electron microscope

Porter· Oh, sure! Why not? This is probably a 210 Ångstrom molecule Every 210 Ångstroms may mark a pronounced coiling of the polypeptide chain, followed by a region of less coiling, a region of hydration, and—

Meyer: But this is really not resolvable by the electron microscope.

Porter: No

Meyer· And that is the question you asked

Porter· Well, I wanted it on the macromolecular level. What is the shape of the fundamental unit? There is the place for speculation to start, if it is going to be clarified.

Wyckoff. I should say that my mind works the other way, though.

I would rather think down from what we see and go just a little way beyond Probably I am not very venturesome in my thinking I don't start from the molecule and think up I start from what we see and think down, and I haven't arrived at the molecules yet

Bauer: Neither have we

György. Is there periodicity in keratin?

Bauer: Yes

Wyckoff. We don't see it

Meyer: In x-rays, there is

Wyckoff. Yes, but we do not see it

Ragan. Has not a fairly definite hypothesis for the molecule of keratin been proposed from x-ray diffraction patterns?

Wyckoff· All the structures for these molecules built upon x-ray data are only possible structures, chosen from a large number of imaginable ones Not enough data now exist to give a unique experimental discrimination between them These structures are nice suggestions only

Meyer· Which have to be modified constantly as time goes on

Bauer. But it provides another approach and, therefore, another way of thinking about the problem

Wyckoff· Yes, but it is extremely dangerous to assume that x-ray diffraction has revealed what these complex molecules are like

Tischel The mathematical probability, though, sets up a certain structure as being more likely to give a particular pattern

Wyckoff But this has not been done It is like the case with the much-studied hemoglobin The people (13) at Cambridge University have been working on its structure for years and they thought they had made real progress in defining its details Then any assurance of this went out the window overnight with Pauling and Corey's (14) suggestions of a new peptide chain But, at the same time, this suggestion is not more than another alternative, although it looks superficially to be more probable than the ones which the Cambridge people were working on

Bauer To do experimental work, you must have a theory to test, right?

Mirsky· Dr Wyckoff is making a very important point, that the tendency occurs too frequently of mistaking the model for the real thing The model is a very valuable tool for further development of hypotheses

Wyckoff The model can help very much in your thinking, but it can also restrict the possibilities that you are prepared to entertain

Mirsky Exactly

Bauer. For your own protection, you should have three models You

could then go from one to another. There is no need to be wedded to one model

Gyorgy: The inventor of the model is obviously biased because otherwise he would not have constructed it. But that is all right and more power to him

Bauer: But we want to know the Wyckoff model

Wyckoff Again, I would say that there are two ways to approach the matter You can start with the smallest imaginable detail, which means starting with atoms in this particular case. From these you can have a model of the molecule. Then you imagine how these are arranged, and so on, with increasing complexity. On the other hand, you can start with what you see and, timidly, move towards the smaller, which is what I said I did.

Meyer: You would first have to go from the atom to the molecule You would have to know the sequence of the amino acids of the collagen molecule before it had any meaning.

Wyckoff: That is quite right.

Porter A macromolecule is an entity in itself. We can forget, for the time being, the polypeptide chain.

Meyer Then how large is a macromolecule of collagen?

Porter. That is what I should like to know.

Meyer: And what is the shape of it?

Wyckoff: Well, I can give you my working picture. It is that of an indefinitely long thread

Porter: Indefinitely long?

Wyckoff: In the same way that a cellulose macromolecule is indefinitely long

Porter Repeating some internal structure every 650 Å?

Wyckoff Repeating every 650 Å, or whatever the maximum is

Bauer: Does it matter where it is repeated?

Porter: It matters to this extent, that there is a 650 Å pattern. Now, Dr. Wyckoff, if it is a thread with a 650 Å repeating unit, how do we get uniform 210 Å fibers?

Wyckoff. It has a substructure, too.

Porter. Yes, but if there is a repeating pattern of density variation at 650 Å intervals, then no matter how the units are put together the macroperiodicity should develop, and sometimes it fails to May it not follow, therefore, that the fundamental unit fibril is constructed of 210 Å units of similar density but dissimilar reactivity and that, to get the 650 Å periodicity, something else combines with this fibril?

Wyckoff: That is correct.

Lillie. Should we conceive of three slightly dissimilar units of 210 Å constituting each unit at 650 Å?

Porter: They are dissimilar, perhaps, in reactivity or in their affinity for mucoprotein or for something else

Wyckoff: Yes, if that isn't purely a fortunate circumstance

Meyer If we assume randomly distributed molecules in this collagen solution, and the crystallization, as we may call it, starts at one point, wouldn't you arrive at a point at which the concentration would be too low to form new fibers, or too low even for the chance of apposition to the ones established?

Wyckoff In other words, you mean that that fiber started off its growth in that center?

Meyer: Yes, and how many centers of crystallization you would have in a field

Wyckoff And we were lucky in having one

Meyer Yes, you were lucky to have detected one

Porter: Yes, but it still follows that there is some influence radiating out from the center to organize along an arc of a circle

György: There was an arc?

Wyckoff No, if it started growing from there and organization proceeded from there, then it would be related to that as a center

Meyer The difficulty is that this process is almost instantaneous, apparently, which makes it a difficult problem to conceive of at all

There is a question I should like to ask you I don't know whether it is along the same line, but what is the similarity or dissimilarity of the salt-reconstituted collagen with the mucoprotein reconstituted?

Wyckoff You don't see this fine structure in the mucoprotein fibrils

Meyer. I think you do

Wyckoff So very fine a structure?

Porter Oh, yes

Meyer At 210 Å, yes

Bauer They do in Boston, unless they put something else under the microscope

Porter Within interperiod striations

Wyckoff Yes, there is some fine structure there

Bauer: Do you think that this reconstitution takes place in nature?

Porter. I don't think that, because it happens, you can infer that it happens in the tissues

Bauer No, I didn't say that by any stretch of the imagination I merely asked the question

Porter In thin sections of tissues, I have yet to see anything resembling a 2200 Å periodicity I think it is a remarkable phenomenon of interaction between mucoproteins and collagen That such a reaction does take place in tissue is probable, but I do not believe that it results in organizations showing those enormous periods

As I recall, it is only when fairly high concentrations of the mucoprotein are used that this extremely large periodicity is found Ordinarily, with low concentrations of mucoprotein, there is 650 Å, just the same as in salting out fibers from acetic acid solutions of collagen

Bauer Edwin J Cohn* thinks that the concentration of mucoprotein added is still within the range one might encounter in humans

Meyer: Because it is diffusible, but its presence in the collagenous tissue has still to be demonstrated When isolated, it undoubtedly is diffusible If diffusible, one might expect it in the urine

Gyorgy. But it is in the urine.

Meyer: Certainly not in high concentrations There may be some.

I wonder whether there is information about the ratio of the soluble collagen to the total, let us say in rat tail tendon This question is concerned with the problem of reconstitution. Can you continue to dissolve a definite quantity of soluble collagen for a given volume of extractant?

Wyckoff. I don't think it can be done indefinitely

Porter Not indefinitely, certainly, but several times?

Wyckoff Yes, you can get more with several extractions, and the amount that you get is definitely a function of the age of the animal

Meyer: I believe Nageotte said embryonic collagen was completely soluble

Bauer: Completely?

Wyckoff: Yes I am pretty sure I remember that.

Bauer: We should do more work with embryonic tissue Why do we play around with adult tissues as much as we do, when we might get along much faster if we worked with the embryonic tissue?

Fischel: Once having dissolved the collagens of embryo and adult tissue and then reconstituted and redissolved them, do you think that it makes any difference what the age of the animal is when you determine the proportion of the reconstituted collagen that dissolves the second time? In other words, in the first solution, you can probably account for a lot of differences qualitatively by age, but after that, I wonder

Wyckoff I don't think we have done enough to say whether or not collagen could be completely redissolved repeatedly, which is what you are asking

Fischel No, you said there was also a residue that will not dissolve That residue may bear the same relationship to the amount in solution after the first step, irrespective of the age of the animal.

Wyckoff. We have no data on that.

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*Personal communication

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THE NATURE OF RETICULIN

A. H. T ROBB-SMITH
*Department of Pathology, Radcliffe Infirmary
Oxford, England*

I EXPECT YOU ALL know a phrase from Whitehead in which he says the scientist, like Humpty Dumpty, enjoys the privilege of paying words extra and making them mean what he likes. In reading the last two Conference Proceedings, one got the impression that these inflationary semantics had caused a certain amount of discussion, and I have been bold enough to suggest a title which I think is liable to induce the same type of reaction, that is "The Nature of Reticulin," or reticular fibrils

Bauer. Is this fact or theory, Dr. Robb-Smith?

Robb-Smith. I think that it is fact at the moment Since Kupffer first described reticulin in about 1876, there has continued to be a good deal of dispute and discussion as to what it is and why it is and so forth Probably the difficulty is that—I wouldn't say all histologists, but it may very well be that Dr. Lillie and I won't agree on the wording—some histologists are reasonably clear in their minds as to what they mean by reticulin, let's say just on the morphologic plane, but there is a great deal of doubt as to precisely what is its relation, on the one hand, to collagen fibrils in the way that Dr Lillie defined them this morning, and, on the other, its relation to chemical collagen or electron microscopic collagen

The difficulty has been to get material which the histologist would accept as reticulin to hand on to chemists or, more recently, to those working with the electron microscope. What can be accepted as the histologic or morphologic definition of reticulin? My reaction is to say that reticulin or reticular fibrils, using reticulin in the morphologic sense and not in the chemical at this stage, consists of fine branching fibers which are nonbirefringent, which do not appear to stain with acid fuchsin in the Van Gieson preparation, which stain with anilin blue in any of the various trichrome stains, and which appear black in a Maresch-Bielschowsky preparation or its modification

Dr Lillie did not seem to be at all happy about the silver methods I must confess I may be lucky or not very critical, but I would say that, taking it by and large, if you have a silver method which you know and love and cherish, it is pretty consistent; in other words, you will find

the fine fibrils are black in untoned preparations and the broader fibrils are brown and are what one would call collagen

Bauer Irrespective of fixative?

Robb-Smith: There is no doubt that the mercuric fixatives do not work nearly so well as formalin; at least, that is my experience The exceptions in silver staining which were mentioned this morning, where occasionally fine fibers are black and so on, are on the whole rare or are from definitely diseased states I am talking at the moment of human material in healthy people

This type of material, I think one would say, merges with morphologic collagen fibers, and here I would not disagree with the definition we had On the other hand, it will not form gelatin on boiling because it has been fixed, denatured Morphologic collagen is coarser, with broad bands of fiber and is birefringent, it stains blue with the anilin blue in the various types of trichrome preparations, red in Van Gieson preparations and in the untoned silver impregnations it is brown I am not bothering about toned ones, because, although they look prettier, they are unreliable and I wouldn't trust them for a moment

As far as the reticulin goes, the fine nonbirefringent, black argyrophil fibers, I would say that they are seen between epithelium and the loose connective tissue everywhere in the body, between blood vessels and loose connective tissue, surrounding muscle fibers, both smooth and striated, around nerves, and, of course, in the true loose connective tissue We need not go into too much detail on that, as it is, so to speak, on the histologic plane

In the work I have been carrying out in association with Harold Kramer, we felt that the first thing was to see whether we could find a tissue in the body in which the connective tissue fibers corresponded to the definitions I have given of reticulin, one in which there was very little collagen or such collagen as there was could be separated from the reticulin If such a tissue could be found, then the next thing would be to see whether that sort of reticulin, histologically and by other methods, was similar to reticulin in tissues where there was a close relationship between reticulin and collagen Reticulin from the particular site or sites that might turn up could then be studied in all the various ways which are available

After looking at a number of tissues in fresh postmortem material, we concluded that the best site from our point of view for getting plenty of reticulin with very little collagen admixed was the kidney in children and young adults, that is to say, we limited ourselves to people about the age of 14, and they were only cases where there was no obvious renal disease It was found that there is quite a bit of collagen in the capsule and around the blood vessels, but, apart from these areas,

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the midcortical connective tissue is almost pure reticulin. Someone is going to say: What about the basement membrane? That is the immediate catch The black fibers were in the same place as the basement membrane and they were part of it This, again, presents a difficulty as to definition Is one going to say that the basement membrane is the homogeneous material apart from the argyrophil fibers, or should it be said that it is the argyrophil fibers apart from the homogeneous material, or a bit of both? I put it to you that the easiest way, because it is very difficult, except by enzymal methods, to separate them, is to say that basement membrane is essentially both; there is a homogeneous material and there is also a network of argyrophil fibers Whether they are the same chemically, and I don't for a moment think they are, is not the point I shall use basement membrane in the sense of both the fibers and the homogeneous material.

When we began to look at other tissues in this way, it seemed to us that reticulin or basement membrane was a matter of degree according to site Tissues like the kidney and muscle, where there is a definite homogeneous material with a nice reticular network, look as though they are closely related, being a membranous type of material in which there are fibrils coursing Tissues like lymph nodes, and spleen to some extent, are mainly fibrillar material, reticular material as I have defined it, with very little homogeneous material; in fact, even in thick preparations, practically no homogeneous membrane can be seen Other tissues are in between these two extremes Liver, for example, is one of the middle ones in which the homogeneous material is not nearly so obvious Thick, frozen sections are necessary in order to be able to see it It has a straplike appearance, with a certain amount of homogeneous material, but the fibrillar pattern is far more striking.

Gyorgy: There is disagreement between you and Dr. Lillie as to the liver

Robb-Sm [illegible]

Lillie: I [illegible] ilars, but I am struck by h [illegible] ethods that he used, what I had to say

Gyorgy. Did you not mention that there was no basement membrane, as you call it.

Lillie Basement membrane material, in the sense that we were able to differentiate it, we could not see in the liver.

Gyorgy: But he sees it

Lillie He interprets some of the silver-positive material which he sees as basement membrane

Robb-Smith: I wouldn't agree with that, with due respect. I was coming in a minute to the question of the periodic acid Schiff reaction,

material is really the thing which up to now I have talked about as this homogeneous material, but there isn't nearly so much of it as there is in tissue like muscle or kidney.

Lillie: We did not say that the collagen and reticulum are periodic acid Schiff negative

Robb-Smith: No

Meyer: But is there any other method by which to recognize the homogeneous substance in the basement membrane, aside from the periodic acid Schiff reaction?

Robb-Smith: I don't know of any that I would regard as a specific method, that is to say, it can be stained in many ways, but all that can be said is that it is being shown up as a color by a particular stain

Bennett: Isn't it true that if you apply, let us say, a Mallory anilin blue stain, you get a blue staining band which we interpret as being connective tissue, whereas if you apply a combined reticulum stain and a periodic acid Schiff reaction, you find a core of interwoven and delicate, blackened fibrils, with a red-staining substance superimposed upon or surrounding them?

Robb-Smith: Yes

Bennett: It has been my impression that the material which stains blue with the Mallory anilin blue connective tissue stain incorporates both of these substances but does not differentiate between them, is that right?

Robb-Smith: I think in general it is true that the Mallory is a less specific method than the silver.

Bennett: Would you agree with that, Dr. Lillie?

Lillie. Yes, I think so

Bennett: And yet it stains the same zone

Lillie: What has been said about the negative Van Gieson stain of the reticulum is true when acid fuchsin is limited to 50 mg. per 100 ml But if the acid fuchsin is stepped up to higher concentration and the specificity controlled by the acid content, the finer fibrils can be stained with the acid fuchsin There is a difference in these fibrils, in the coarser ones, but it is a quantitative rather than a qualitative difference, I believe, in that particular respect I do not say that there are no other differences

Fremont-Smith: Using the same method, would each of you actually disagree as to what would be seen in terms of color and size?

Lillie. Prior to our offering this new variant, I believed essentially what Dr Robb-Smith is saying, on the basis of the same sort of evidence

Bennett What about the silver story?

Lillie We had never previously combined the silver with the periodic

Holbrook. But you took the position this morning, Dr. Lillie, that the silver staining was by no means—

Lillie Limited to reticulum.

Holbrook: That's right, and that it involved the collagen fibers sometimes strongly and sometimes lightly. I believe I understood Dr. Robb-Smith to say that he makes quite a distinction between the reticular and the collagen fibers; is that correct?

Robb-Smith· Yes.

Fremont-Smith· By silver?

Holbrook· By silver.

Lillie. With regard to Dr Robb-Smith's uniform success with Maresch-Bielschowsky, I can only congratulate him I have had very different results on serial sections off the same block of kidney that were put through simultaneously In regard to the amine silver methods, most of us have used more than one method, and because we happened to run into a percentage of success with a certain one, we have decided that that was the best one, whereas our colleague across town swears by all that is good and holy that another one is very much better than the one we are using

Dempsey May I illustrate this difficulty with a couple of stories? One of them is that I had a young man working with me this year who had come directly from the Cajal Institute, where he had been working with silver methods and had been very successful with them He had the slides with him to prove how successful he had been I asked him to prepare some slides in my laboratory to show me how to do it He failed totally, using the same method that he had used in Spain

Fremont-Smith Supposedly the same.

Dempsey Supposedly the same, so far as he knew He then modified the technique quite markedly and succeeded. His explanation was that the water in St. Louis was very different from that in Madrid

Gyorgy That's right, it is.

Dempsey. The second story is about a reticulum stain For fifteen years I used a modification of the Bielschowsky stain known as Pap's method It was a standard method in our laboratory at Boston I went to St Louis and it failed me I began to cast around and I ended up with a modified Wilder, which is similar in theory but different in technique, and it works fine So there are differences in the hands of the same investigator

Holbrook That is a good point.

Dempsey This makes it understandable as to why we disagree slightly on technique

Lillie: We have not yet arrived at the point in the control of silver stains where we can make them constant throughout the world. One of the best witnesses to that is the vast multiplicity of silver methods in the literature. However, if there had been a good one in the first place, it would be used all over.

Robb-Smith: These are methods which must be done with loving care. The reticulin ones are a little less tricky than some of the others, but they too can present pitfalls. I had an experience rather like Dr. Dempsey's when I was working with Hortega in Spain. There were two of us, an American and myself, and we were having trouble with oligodendrocyte impregnations. He could get it to work and I couldn't. We cut serial sections from the same frozen block, put them into two glass dishes, and so forth. It was as near as possible a duplicate experiment. The only difference was that he was working on the north side of the room and I was working on the south side. The result was that one of us could make it work and the other could not. Nonetheless, Dr. Dempsey and Dr. Lillie have illustrated that once you get a method which you speak nicely to, 99 per cent of the time it will work on normal material.

Fremont-Smith: For you.

Robb-Smith: For you, in your laboratory.

Holbrook: And if it works for you, it is much more important than if it doesn't work for the other fellow.

Robb-Smith: Yes, because he will have another method that he can talk to.

Fremont-Smith: The artistic element in microscopic staining must be acknowledged. In the future, one must say, "My method is fine provided you are an artist and take loving care. Don't expect to be able to reproduce it easily and don't throw it away because you can't."

Robb-Smith: Possibly, we might get one point clear. Wouldn't Dr. Dempsey and Dr. Lillie agree with me that when things are working well, the sort of things which I have said are more or less true, with his method or mine? I have not defined what my method is, but it doesn't matter.

Dempsey: It doesn't matter. We would agree.

Robb-Smith: We might look at some not particularly beautiful preparations of silver stains in different tissues before we go on to the next stage. Figure 33 is a section of spleen. The fine branching fibers, which would be argyrophil, which are nonbirefringent, and which stain with anilin blue, and so forth, can be seen. My word will have to be taken for this but there are fine black fibers and coarser brown ones, which give the pattern of appearance in the spleen of collagen and reticulin fibers, emphasizing the point that it is possible to trace a con-

FIGURE 33 Human spleen (reticulin impregnation). The interrelationship between the fine reticulin fibrils and the coarser collagen fibers can be seen

tinuum from quite fine branching fibers to where they merge imperceptibly with the larger fibers which would be regarded as collagen ...tion. Again ...oarser fiber ...ones This is a definite sort of membranous effect. Figure 35 shows striated muscle which has been treated with trypsin and emphasizes this membranous effect This is an argyrophil preparation and is the same sort of thing seen in kidney.

Those are random samples of the different types of reticulin, the fine, fibrillar one without very much membranous material, as in spleen; the straplike ones, which are seen in the skin; and the membranous type of which kidney and muscle are examples.

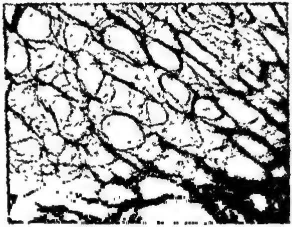

FIGURE 35 Human muscle (reticulin impregnation) The section was treated with trypsin solution and the muscle fibers have disappeared It is a tangential section Reprinted, by permission, from Robb-Smith, A H T Tissue changes induced by Cl welchii type A filtrates *Lancet 2, 362 (1945)*

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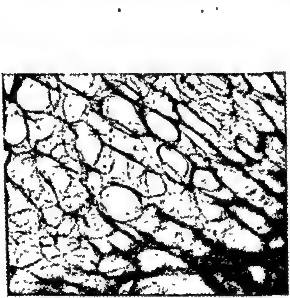

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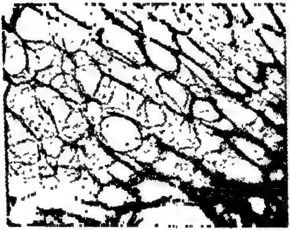

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Lillie I do not believe in the original experiments that they used anything like stoichiometric proportions. There was a considerable excess of periodic acid and over a very much longer time exposure than we had been using, histologically

Meyer When measuring periodic acid oxidation, one usually titrates to a point where there is no longer an appreciable uptake

Lillie I am not speaking of the present-day titrations of periodic acid I am speaking of the original experiments of Jackson and Hudson in which they used excesses and gave long exposures to allow the reaction to go to completion There was no mention of the actual consumption at that time That came later

Fremont-Smith I should like to make a general comment Dr Robb-Smith is saying that if one waits longer, they will stain the same, and Dr Lillie points out that with a wait of a shorter time, they will stain differently One of you gives significance to the sameness and the other one to the difference This parallels an incident with apples Somebody came along and said, "Why, these all look alike and we will call them apples If you really look at them, it can be seen that they are all the same Examine them and they have the same chemical constitution Watch them as they ripen and they go through the same chemical changes They have the same waxes in the skin, they taste the same, and so forth" Somebody else came along and said, "This is perfectly ridiculous Just take any two apples off the same tree, off the same branch, and measure them accurately in any respect, whether it is weight, amount of carbohydrate, enzyme content, thickness of skin, number of seeds, and a difference will be found in every one of them The significant thing about these two apples is that each one is different from the other in every respect that can be measured"

This is an illustration of the kind of place where a discussion founders One group is paying attention to the similarities and the other group to the differences Obviously, both are correct, both are meaningful, but they are meaningful in different ways Our job, if we were to push this to a conclusion, would be to say what is the significance of the fact that Dr Robb-Smith, on the one hand, can get the same stain if he waits, and, on the other hand, Dr Lillie can get a different stain if he doesn't wait

Robb-Smith I am content to leave it at that Using the method as we do, my feeling is that where there is obvious membranous material *in relation to the argyrophil fibers, there a good positive PAS can be* obtained Where very little membranous material can be seen by a nonspecific method, there will also be very little PAS There seems to be some parallel between the amount of nonargyrophil membrane, which was closely related to the argyrophil fibrils, and the positive PAS reaction

Coming to the question of the periodic acid Schiff reaction, I would say—and there may be shouts of protest here—that if the ordinary method is used, with possibly a lot of sulfite about, coarse collagen fibers do not stain They stain a yellowish-red color.

Lillie: Is that with alcoholic periodic acid?

Robb-Smith: Yes.*

Lillie: That is much slower to react than the aqueous.

Robb-Smith: Yes On the other hand, in the sites where these fine reticulin fibers are located, a bright red color is obtained. In other words, there is a striking difference between things which are morphologically or histologically collagen and things which are in the site of morphologic reticulin

Lillie. Our experience has been that it takes about two hours to effect as much oxidation with strong alcoholic periodic as is gotten in five or ten minutes with an aqueous solution of the same strength, so I would say that that failure to demonstrate collagen is due to underoxidation

Robb-Smith. A consistent difference can be obtained

Lillie A consistent difference is found by interrupting the aqueous oxidation in a matter of seconds.

Fremont-Smith. But isn't that difference significant in itself?

Lillie: Oh, yes

Fremont-Smith. If one oxidizes much more rapidly than the other, it means a difference.

Lillie. Or that one has many more reactive groups than the other

Fremont-Smith. Well, that is the difference.

Meyer Is this chemically on a firm basis? When periodic acid oxidation is used in 1-2 glycols or amino alcohol, it is usually done under well-defined and as mild conditions as possible If these conditions are not kept, periodic acid might oxidize groups which are not necessarily 1-2 glycols

Lillie. However, Hotchkiss (2) did not point out this difference between the alcoholic and aqueous periodic acid in his 1948 paper.

Meyer. We should be careful about the interpretation of results based on periodic acid oxidation

Lillie The aqueous oxidation which we have used is far milder than that which Jackson and Hudson (3) employed on starch

Meyer: I don't recall what Jackson and Hudson used for starch, but it has always been stressed, as with any oxidant of this nature, that there must be a very slight excess of the periodic acid. In starch, since the number of hydroxyl groups is known, it can be calculated what the excess would be

Lillie. I do not believe in the original experiments that they used anything like stoichiometric proportions There was a considerable excess of periodic acid and over a very much longer time exposure than we had been using, histologically

Meyer. When measuring periodic acid oxidation, one usually titrates to a point where there is no longer an appreciable uptake

Lillie I am not speaking of the present-day titrations of periodic acid I am speaking of the original experiments of Jackson and Hudson in which they used excesses and gave long exposures to allow the reaction to go to completion There was no mention of the actual consumption at that time That came later

Fremont-Smith: I should like to make a general comment Dr Robb-Smith is saying that if one waits longer, they will stain the same, and Dr Lillie points out that with a wait of a shorter time, they will stain differently One of you gives significance to the sameness and the other one to the difference This parallels an incident with apples Somebody came along and said, "Why, these all look alike and we will call them apples If you really look at them, it can be seen that they are all the same Examine them and they have the same chemical constitution Watch them as they ripen and they go through the same chemical changes They have the same waxes in the skin, they taste the same, and so forth" Somebody else came along and said, "This is perfectly ridiculous Just take any two apples off the same tree, off the same branch, and measure them accurately in any respect, whether it is weight, amount of carbohydrate, enzyme content, thickness of skin, number of seeds, and a difference will be found in every one of them The significant thing about these two apples is that each one is different from the other in every respect that can be measured"

This is an illustration of the kind of place where a discussion founders One group is paying attention to the similarities and the other group to the differences Obviously, both are correct, both are meaningful, but they are meaningful in different ways Our job, if we were to push this to a conclusion, would be to say what is the significance of the fact that Dr Robb Smith, on the one hand, can get the same stain if he waits, and, on the other hand, Dr Lillie can get a different stain if he doesn t wait

Robb-Smith I am content to leave it at that Using the method as we do, my feeling is that where there is obvious membranous material in relation to the argyrophil fibers, there a good positive PAS can be obtained Where very little membranous material can be seen by a nonspecific method, there will also be very little PAS There seems to be some parallel between the amount of nonargyrophil membrane, which was closely related to the argyrophil fibrils, and the positive PAS reaction

I had been interested, left-handedly, *in reticulin for some time,* and I became rather excited about it when playing about with gas gangrene in the war. I became interested in some of the toxins, which one could also regard as enzymes, which these organisms seem to produce. In doing enzymatic analyses of tissues, particularly of the collagenous group, it is vital that they not be fixed in formalin because formalin does curious things to collagen, which I don't understand but the chemists do. You cannot work properly with these enzymes after it, and so we always use alcohol or Carnoy fixation and various tricks in applying the enzymes, which don't really matter at the moment.

At that time our feeling was that the basement membrane of the kidney was the tissue which we were going to be able to get free of collagen, but we were trying to compare it with reticulin in other tissues. We had the impression by other staining methods that there were simply differences of degree between kidney reticulin and, for example, spleen reticulin. We therefore went on to try enzymes. Apart from the standard method, we have always, as controls, exposed sections to the buffer alone, the boiled enzyme with buffer, and sometimes with an antitoxin, if it is available. With pepsin, there is destruction of both collagen and reticulum and most of the tissue, and the result is horrible to work with. With trypsin, using crystalline trypsin, one can get perfectly good silver staining and also a positive periodic acid Schiff, but it gets rather diffuse. It is untidy, not nearly so clean as before *trypsin. Figure 35 is of a muscle after trypsin.*

Hyaluronidase, we found, had no effect on periodic acid Schiff staining, or silver. There is a change in pattern, the tissues tend to loosen up quite often, but there is no change in the silver reactions.

With collagenase* one finds that the silver-positive material, both black silver and brown silver, disappears and, also, the material becomes PAS-negative.

Parenthetically, I might say that collagenase is in no way new. Mall used it; at least I am quite certain he did. *He used some organisms* that he had found in the garden and cultured; there is little doubt *from the reactions* described that he used a crude collagenase in his studies on reticular fibrils at the end of the last century.

Figure 36 is merely a reticulin preparation of muscle, and *it is impressive in* that the reticulin has not stained. It may very well be that Dr. Lillie or Dr. Dempsey will say that it is just the method that has gone wrong, but there it is.

Dempsey: We are not that impolite!

*The collagenase used in these experiments was a purified Cl. welchii filtrate *neutralized* with antihyaluronidase, anti-α and anti-θ antitoxin. It would seem *from the reports of certain* workers [Gersh and Catchpole (4), Stoughton and Lorincz (5)] that their *preparations* of Cl. welchii filtrate had little collagenase activity.

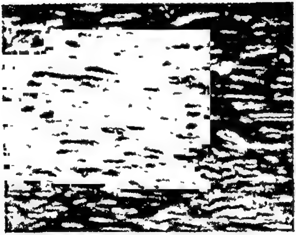

FIGURE 36 Human muscle (reticulin impregnation). The section was treated with collagenase solution and the reticulin membrane has disappeared, although the muscle fibers are unaffected Reprinted, by permission, from Robb-Smith, A H T Tissue changes induced by Cl welchii type A filtrates *Lancet* 2, 362 (1945)

Robb-Smith An odd thing turned up when we were gradually wandering through tissues, using the collagenase Very tiresomely, we found some fibers which were argyrophil-positive, collagenase-resistant, and destroyed by trypsin These fibers are present in the ovary We haven't found them to be birefringent, and they are, so far as we can make out although I wouldn't like to be definite about this, PAS-negative; this could only be checked in sections which had been treated with the enzymes, as in no other way could they be distinguished from true reticulin

Gyorgy The ovary is the only organ in which you found them?

Robb-Smith Yes!

I had intended to keep off the pathologic types of connective tissue, but pathologic connective tissue, or, at any rate, abnormal connective tissue, can be found, for example, in the corpora albicantes, which, after collagenase, is Masson anilin blue positive, but has no collagen in it from the point of view of argyrophil material In toluidine blue preparations, the aniline blue positive areas are metachromatic, and if the sections are treated with hyaluronidase, the metachromatic material goes and the areas stain a grey-blue with aniline blue If sections are treated with trypsin, the whole lot can be knocked out, including the blue Well, that is just a little by the way

In Figure 37A, a close-up of a bit of ovary, a control reticulin prepara-

FIGURE 37 B.

FIGURE 37 A.

FIGURE 37 A Human ovary (reticulin impregnation). *Fine argyrophil fibrils* can be seen in the stroma and around the blood vessels, and the "hyalin" of the corpus albicans shows argyrophilia.
FIGURE 37 B Human ovary (reticulin impregnation). This section from the same block has been treated with collagenase There is no staining of the perivascular connective tissue or the corpus albicans, but the fine argyrophil fibers of the stroma are still present.

FIGURE 40 FIGURE 39 FIGURE 38

FIGURE 38 Human ovary (reticulin impregnation) The coarse argyrophil material of the tunica albuginea contrasts with the finer fibrils of the medullary stroma

FIGURE 39 Human ovary (reticulin impregnation) This section, which was from the same block as Figure 34, has been treated with collagenase There is no staining of the tunica albuginea, but the fine argyrophil fibers of the stroma are still present

FIGURE 40 Human ovary (reticulin impregnation) This section, which was from the same block as Figure 38, has been treated with trypsin The collagen and reticulin fibers of the tunica albuginea and the perivascular stroma are still present but the argyrophil fibrils of the medulla have disappeared

tion, and the blackish or the brown collagen fibers can be seen round about the blood vessels, and the rather fine-looking network which one would accept as branching fibers of reticulin.

Figure 37B is of a postcollagenase section; the portion of the corpus albicans is unstained, but there are still fine fibers These are the argyrophil collagenase-resistant fibers.

Figure 38 is of cortex of the ovary, a reticulin control, with the dense, collagenous material and fine black argyrophil fibers.

Figure 39 is a collagenase preparation; the collagen outside has gone, but the fine fibers still stain with silver

Figure 40 is a trypsin preparation The collagenous material in the cortex and the collagen around the blood vessels stain, but the fine argyrophil material which is collagenase-resistant has disappeared. I mention this to illustrate that one can, on occasion, be caught out by some of these enzyme methods; in the ovary there are fibers which, unless enzyme methods are used, fulfill all the criteria which I mentioned earlier for reticulin However, when enzyme tests are applied, they are found not to be reticulin What they are and where they come from, I am not prepared to go into now.

Taking it by and large, it seemed to us that the type of material which we had accepted as reticulin on morphologic grounds did have definite characteristics from the point of view of these enzyme methods, and so the next thing was whether we could go on to study it from the point of view of chemistry and of electron microscopy. This conference was coming on and we did not really have time to finish the work before I came over, a good deal of this has to be tidied up, particularly as to the chemistry But what we did was to take kidney, strip off the capsule, cut it into thin slices, and then remove about 2 mm of the subcapsular cortex, the blood vessels were punched out with a corkborer. We removed the medulla and about 2 mm of juxtamedullary cortex, leaving only mid-cortical tissue, making quite certain that we had not included any medulla

A histologic check of that material showed very, very little collagen, as I have defined it, and that there was quite a lot of reticulin as well as basement membrane. We treated these thin slices with crystalline trypsin and got the same sort of preparation as for muscle It was not very clean, there was usually a bit of debris left in spite of repeated washings, and so on However, it behaved perfectly well from the point of view of the various staining methods and enzymes and so forth, already described Then we tried using decinormal sodium hydroxide at room temperature and found that that gave a very clean preparation The actual technique used was to take frozen sections of this material, cut at about 5μ, put them in the decinormal sodium hydroxide and

shake them fairly gently over about eight hours, wash, and do a further treatment in the same way

As Dr Lillie mentioned earlier, there was at one time a certain amount of dispute as to whether this particular method of treatment, that is to say, of cold sodium hydroxide, destroyed reticulin fibers The French school said that it did not On this occasion, we are with the Europeans and against the Americans We could get quite reasonable preparations with this method

This material, which at every stage we controlled by examining

FIGURE 41 Human kidney Electron micrograph of reticulin, unshadowed, to show the laminated appearance

stained sections, was used for chemical and ultrastructure studies The x-ray diffraction and electron microscopic material was examined by Dr Little of the Atomic Research Station at Harwell, and I am grateful to her for allowing me to show these preparations. With the x-ray diffraction, one can make out the three collagen rings; those at 4 5 Å and 11 Å are strong, but that at 2 9 Å is weak, apparently because there is a diffraction halo around it

There is a Metrovic EM-2 apparatus at Harwell, and material was examined on a 3 mm copper grid or a copper ring dipped in celloidin

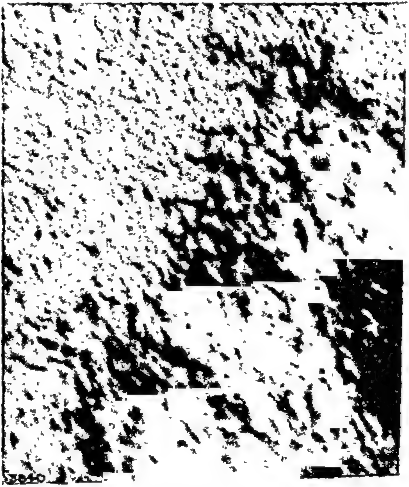

FIGURE 42 Human kidney Electron micrograph of reticulin, shadowed with gold and palladium, to show the interlacing mat of very fine fibers

On the whole, better preparations were obtained with the copper grid The shadowed preparations are with gold, palladium alloy, or uranium If an unshadowed preparation is examined, sometimes there is a quite definitely mottled appearance, and at other times it is more uniform, as Figure 41 shows

In shadowed preparations, for the most part there is an irregularly arranged feltwork of fibers varying in size, a lot of them very small, down to about 100 Ångstrom broad We have been able to make out the 650 periodicity in them, but they are quite randomly arranged In

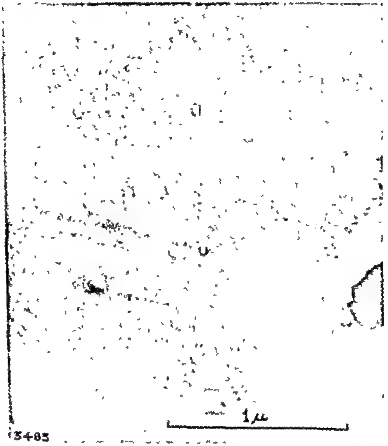

FIGURE 43 Human kidney Electron micrograph of reticulin, shadowed with uranium, showing the network of fine fibers and an occasional larger fiber with a clear periodicity

Figure 42 can be seen the meshwork of irregular fibers, some slightly larger than others, with no large fibers in them.

Figure 43 is a preparation where somewhat larger fibers can be seen, but again there is a completely random arrangement, a random background.

Wyckoff: Are there any 650 A striations there?

Robb-Smith. That is hardly in focus, but some cross-striations can be seen.

Figure 44 is another where an occasional, quite large fiber is seen,

FIGURE 44 Human kidney Electron micrograph of reticulin shadowed with gold and palladium, showing the feltwork of fine unorientated fibers together with larger orientated fibers

but most of the fibers are really very small and they are scattered about in this partially amorphous, partially fine fibrillar-patterned background That was the appearance of the preparations of kidney for the most part, a meshwork or network of irregularly arranged, very fine fibers, with occasional, quite definite, larger fibers which would ordinarily be classed without much hesitation as being of the collagen type

FIGURE 15 Human adrenal Electron micrograph of connective tissue, shadowed with uranium showing the range of fibers from the fine feltwork to large orientated fibers

When we took the other material, such as adrenal, muscle, and so forth, which we treated in the same way but which we knew was contaminated—if it can be put that way—with collagen (Figure 45), in the background can be seen the same rather amorphous feltwork of irregularly arranged fibers. A curious thing in a number of these preparations is the impression of areas where there are lacunae about 500 Ångstroms in diameter. We don't know by any means whether or not it is an artifact. There is one just a bit above the bottom of Figure 45.

FIGURE 46 Human adrenal. Electron micrograph of connective tissue after boiling, shadowed with gold and palladium. The larger fibers have disappeared, leaving their outlines, but the background shows a mottled appearance.

When these preparations are lightly boiled before they are put on the electron microscope grid, we find that the coarse fibers go and there is left the sort of appearance which is shown in Figure 46 While there is no doubt that the ones treated with collagenase are, so to speak, incompletely exposed because if left for too long nothing remains to put on the grid at all, treated for a short while, the classical collagen fibers go first, and the mesh or feltwork background persists a little longer

This is really about as far as we have gone The chemical methods which we have used are extremely primitive If unfixed sections of kidney prepared in the way I have described are boiled, they break up but do not go into solution and no significant amount of gelatin is obtained It does not behave in any way like satisfactory preparations of collagen from the point of view of boiling, in that there is a very considerable residuum left behind If boiled with hydrochloric acid, the result is disintegration but no solution If boiled with sodium hydrate, it goes into solution in no time After boiling in water, the insoluble material gives crude reactions for carbohydrate and peptides, no tyrosine or tryptophan can be detected, and no significant amount of sulfur, and, using MacFarlane and Guest's method, strong reaction for hydroxyproline is obtained With the collagenase-treated sections, there is complete solution As yet, we have not studied the nature of the filtrate in which the fibrillar element is dissolved

Our feeling is that morphologic reticulin, as we have defined it so far, obviously contains collagen from the point of view of ultrastructure, but it appears to be closely related to some carbohydrate material Whether it is actually bound in some way we do not know, although some of our experiments suggest that it isn't just a matter of the two things lying together like jam between two pieces of bread, there is probably a closer relationship than that All that can be said is that we have tried to study material in which there has been very little histologic collagen, and that has been to a large extent reticulin-basement membrane or basement membrane plus reticulin, according to how you look at it

Holbrook Dr Robb-Smith, would you review again the three or four significant differences, as you see them, between reticulin and collagen?

Robb-Smith The first thing is that, taking it at the visible light microscope level, in reticulin the fibrils are finer than collagen fibrils, they branch, and they are not birefringent, collagen is the opposite side of the penny as far as these things are concerned From the point of view of staining methods given that you have a Maresch-Bielschowsky method that you are used to, fine fibers are black and the colla fibers brown, but, as I mentioned, there will be intergrades between

two. As far as the PAS staining is concerned, I would say, by the way we have done it, that collagen is negative and that the fine fibers are probably negative, but that they are always very closely related to positive PAS material. From the standpoint of enzymatic studies, the main difference is that the pectinases and things like that do not do anything much to collagen, whereas they do attack the argyrophilia and the PAS positiveness, but whether the fibers, in fact, have disappeared or whether it is just a change in staining reaction, I intend to be a bit shifty about

As far as the other enzymes are concerned, there are similarities, in other words, there are identities rather than differences The collagenase breaks down both reticulin and collagen, and trypsin and hyaluronidase do not alter either type of fiber. From the point of view, so far as we have gone, of electron microscopy, in preparations of almost pure histologic reticulin, there is this rather random feltwork of fibers which appear to be rather thinner than most, or, at any rate, their range is narrower than the usual range of collagen fibers but they have the periodicity of collagen; in other words, their pattern is different rather than their structure

Holbrook: Have you any idea what their origin is? Do they originate from collagen fibers, from ground substance, or from a totally different source?

Robb-Smith: I haven't any idea, but I should be very content to believe that, given the right building stones—and whether those come from serum proteins or what is, I think, anybody's guess—then presumably there will be some enzymes which will build them up or orient them so that there begin to be polypeptides pretty closely related to collagen I think it is very likely that in the first stage, in the reticulin, there is some bonding between carbohydrate and the polypeptide chains; then, as the thing gets more mature, or more like morphologic collagen, probably it reduces its relationship to carbohydrate, but that is pure guesswork

Dempsey. But you believe they are functionally and morphologically continuous with the collagen fibrils, that is, that there is a direct morphologic continuity between the two classes of fibers?

Robb-Smith: Oh, yes, but that they are different

Ragan Would you say that the reticulum branches into the collagen?

Robb-Smith. It is like a river joining the sea

Meyer. Have you done experiments in which these kidney slices were treated with citric acid in order to see whether there is any specific difference in the solubility?

Robb-Smith: No, we haven't done that

Meyer: That might be quite helpful.

Robb-Smith: From the point of view of getting the carbohydrate?

Meyer: No, not of carbohydrate, but of procollagen of Orekhovich

Lillie: The gelatin or gelatin-like substance which Mall reported from reticulum was derived, I believe, from intestinal mucosa rather than from kidney, so there are perhaps a multiplicity of reticulums as well as collagens

Robb-Smith But, on the other hand, both in lymph nodes and intestine there is a good deal of collagen You see, that is the difficulty, *that there is a good deal of morphologic collagen*

Lillie: Your Maresch-Bielschowsky was the silver nitrate pretreatment, before the amine silver Do you happen to know what the pH level of your silver nitrate solution was?

Robb-Smith: I couldn't answer that, I am afraid

Lillie We made one the other day We happened to check and it was around 8, much to my astonishment I thought it would be on the acid side

Bennett: Have you utilized these methods of observation on the glomerulus itself?

Robb-Smith: No, we have not, we have stayed clear of it My own feeling, and I think there is a certain amount of evidence for it, is that glomerular basement membrane is a bit different from ordinary basement membrane We got into enough trouble over those funny fibers that we found in the ovary

Lillie We are essentially in agreement on the periodic acid Schiff reaction; we have been getting what we recognize as a weaker reaction in collagen and reticulum than in basement membrane, and you, by attenuating procedures, have gotten no reaction

Robb-Smith: Yes, that is right

Lillie There has not been enough recognition of the fact that the Hotchkiss technique is a quite different technique from the McManus, for example People have just blithely said "Hotchkiss negative," and one never knew which they meant or whether they used the Hotchkiss B

Ragan. Do these reticular fibers swell on acidification?

Robb-Smith As yet, we haven't really examined them when unfixed, which is the only way to do it, but most people have said that they do not

Holbrook If they do not, that would be another difference between collagen fibers and reticulin

Robb-Smith Yes

Meyer I assume that the pectinase from onions is not very defined Did you mention whether this same material is also metachromatic?

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Meyer I assume that the pectinase from onions is not very defined Did you mention whether this same material is also metachromatic?

Robb-Smith: No, it is not Ordinary basement membrane is not metachromatic, I would say

Lillie. Have you seen McManus's (6) report on pectinase?

Robb-Smith. Yes. He used polygalacturonidase.

Lillie His results did not differ greatly.

Meyer. Yes, I remember that The source is not onions. This is a commercial pectinase from the brewing industry; I believe it is a mold product

Robb-Smith I wouldn't be in the least proud of it as a precise method It is one of those glorious bits of muck that you put on, and all you can say is that, if you do it under certain conditions, something happens

Meyer. And the hyaluronidase was testicular hyaluronidase?

Robb-Smith Yes, testicular

Lillie We have done essentially the same experiment and had no results from prolonged bovine testis hyaluronidase action on renal stroma

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HYPERSENSITIVITY AND THE HYPERADRENAL STATE*

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To be discussed are some of the aspects of hypersensitivity, particularly as they relate to, or as they are altered or not altered by, the hyperadrenal state. In this presentation, there will be a change in orientation from the cellular level with which the previous discussion was concerned, and the phenomena will be viewed on the tissue level and from the point of view of the organism as a whole. Although what few studies have been made show that immunity is a property of cells, perhaps just as respiration and metabolic activity are, my own work in the field has not been on the cellular level. I hope we will have some discussion later on fibroblasts and other cells that are endowed with the properties of immunity.

There is a reluctance to consider allergic phenomena from a scientific point of view because allergy is a very broad field and encompasses so many variables. The subject has an extensive literature, much of which is uncritical. In part, this is because the data are difficult to obtain in pure form, or to study as isolated examples with only one or, at the most, two variables. Almost every experiment in allergy entails an antigen or antigens, usually proteins foreign to the animal into which they are injected and constituting the first of the many known variables. Antibodies are produced by the host, and there is evidence that in some instances there are various types of antibodies specific for a single antigen. The host produces antibodies in widely different amounts within the same species. The amount produced has a definite bearing on the extent of a necrotizing allergic reaction or of a wheal and erythema type of reaction. After the antigen-antibody union is effected, different degrees and different types of tissue damage may result in different areas of the body, and vary from species to species. Most contradictions in the literature can be explained on the basis of the fact that many investigators use complex antigens, horse serum or egg white

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* This work was aided by grants from the Helen Hay Whitney Foundation and the Masonic Foundation for Medical Research and Human Welfare.

which preclude quantitative appraisal of their results, and many do not appear to be aware of the fact that antibody production can be so extremely variable within a single species.

One further word, namely, that the word "quantitative" as applied to much of the research done today has been used loosely in many respects. In contrast to others, studies of the type initiated by Drs Heidelberger (1) and Kabat (2, 3) have brought to immunology the critique and exactness, the precision which is present in quantitative chemical analysis A single antigen, that is, a single chemical and not a mixture, has been found to react with its homologous antibody in a certain well-defined fashion which can be expressed mathematically The amounts of these reagents which interact are related to the severity of certain of the biological reactions which occur *in vivo*.

There are still publications, and one of them is the *Transactions* of a Macy Conference of a year or so ago, which speak of quantitative studies when what is meant is a statistical approach, that of using complex antigens such as horse serum in response to which varying and indeterminable amounts of antibodies are produced The thinking here seems to be that if enough animals are run, an answer may be found However, with the number of variables in this topic, the error is also compounded to a great degree, so that the statistical approach leaves something to be desired unless a well-standardized system is employed Of course, we all hope to employ statistical analyses of our results, provided they are not arrived at with a hodgepodge of agents As an analogy, there are still some difficulties in the standardization of digitalis Using a leaf preparation as the standard, the cat or frog heart's reaction to a particular leaf preparation had to be statistically appraised, but there is no certainty that the effective ingredient in the leaf preparation is constant from year to year or from animal to animal A pure chemical could be evaluated statistically, with more probability of obtaining reproducible results. A well-defined chemical could be established as having an effect and could serve reasonably as a standard from year to year without variation

The field of hypersensitivity—I use the terms "hypersensitivity" and "allergy" interchangeably—has recently received great stimulus from studies on the adrenal hormones. However, the literature goes back well into the twenties and thirties when crude adrenal extracts were used in different types of allergic reactions

At the outset, it is important to note that the amount of antibody involved in all allergic reactions has a good deal to do with the severity of the reaction (4). Dr. Kabat and various of his co-authors have established the fact that in anaphylaxis, for example, the reaction of a sensitized guinea pig to the injection of a foreign protein, such as crys-

tallin egg albumin, is largely dependent on the amount of antibody present in the guinea pig For this, the technique of passive immunization was used, since active immunization results in the production of an unknown amount of

15 micrograms of an

with a small dose of

erally die in a state

shocking is not as critical to the outcome, within limits, as is the amount of antibody The symptoms of anaphylaxis, a sudden, dramatic affair, appear within a few minutes There may be mild reactions with itching and scratching of the nose or ruffling of the fur, but shortly thereafter, with increased spasm of the bronchial tree and suffocation, convulsions and death follow

The necrotizing allergic reactions, which may, perhaps, be of more interest in a conference on connective tissues, are those which result in localized death of tissue The classical example in immunology is the so-called Arthus reaction, the reaction described in 1903 by Arthus, who found that the repeated injection of horse serum into a rabbit, although at first appearing relatively innocuous, ultimately resulted in an explosive inflammatory reaction, with edema, cellular infiltration, hemorrhage, and sloughing out of tissue, or death Since then, the definition,

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replete with examples

of Arthus-like reactions in every organ of the body, attempts to produce appendicitis or focal necrosis of the brain or glomerulonephritis, etc If the pattern of the Arthus reaction is extended to systemic reactions, the result is what is classically known as 'serum sickness' in humans and varying degrees of panarteritis If a foreign protein is injected into the vein of an animal or a human, and if there is enough of it remaining after two weeks or so, antibodies are formed which react with persistent antigen to give a widespread inflammatory reaction

György Do you think that the Arthus phenomenon and the Schwartzman phenomenon are interchangeable?

Fischel Not at all

György But from your definition it would almost appear so

Fischel No, the Schwartzman phenomenon does not involve specific antigen

György But necrosis results when something is injected

Fischel. I thought my definition included the union of an antigen with its antibody

György But it is not known, clinically, when an injection is made, whether the union has occurred We clinicians use necrosis to deter-

mine the Arthus phenomenon When something is injected and necrosis results, an antigen-antibody reaction is postulated, but we can never be absolutely certain, clinically

Fischel: There is a difference

György: I know there is a difference, but I would like to have it put in the record

Fischel: The difference is this: In the Schwartzman reaction, the period between the so-called sensitizing dose and the challenging dose is only a day There is hardly time for the production of a specific antibody. Dr Schwartzman has expressed this point very well. The reaction seems to be some sort of summation of toxicity. We may get into a discussion of the so-called Auer phenomenon later, the localization of suboptimal doses of toxin in previously damaged sites In patients with certain types of drug reaction, if that is what you are thinking of, when a reaction occurs several days after the initial injection, it may perhaps be of the Arthus type When the reaction occurs immediately, unless it is known that the patient has been sensitized previously, I agree with you that we do not know whether an antigen-antibody reaction has occurred. There certainly are toxin reactions. Reaction to erythrogenic skin toxins are not Arthus reactions; they are immediate responses to the toxicity of the substance which is injected

György: I was only afraid that, in your broad definition, everything leading to necrosis would be an Arthus reaction, and I thought it was especially important that the Schwartzman phenomenon be separated

Fischel: It certainly should be since the mere similarity of certain of the morphologic end points does not identify the mechanisms which produce them. Many substances and combinations of substances cause necrotizing inflammation, but the union of antigen and antibody is, by usage, the characteristic feature differentiating Arthus reactions from other types As I said, two prototypes of allergic reactions have been fairly well defined according to the techniques of immunochemistry, using known amounts of single substances and their homologous antibodies (4) Much of the literature on the effect of the adrenal hormones on sensitivity reactions involves the use of actively immunized animals Actively immunized animals contain unknown amounts of antibody. Therefore, it might be well to discuss the production of antibodies per se, and how that [illegible] then go on *to allergic reactions induced* [illegible] discussion, I think we shall [illegible] tomized animals It is a well-known and old observation that adrenalectomized animals, just as Addisonian patients, are *highly vulnerable to stress situations; that is, they react* with a fatal effect more often than do normal individuals. Susceptibility to anaphylaxis, or histamine

shock, may be expected to be increased by adrenalectomy and decreased by substitution therapy.

by Dr. the ef- under physiologic conditions but the pharmacologic action of an excess of these hormones, just as an excess is required for the suppression of symptoms in various diseases fulfilling a need of the patie mone comparable to substitu

There are several types of vious experiment is that in which there are two series of animals, both of them immunized simultaneously, but only one group treated with ACTH or cortisone Simultaneous control studies are necessary because, even with the most precise techniques, variables are probably present which cannot be appraised Therefore, we cannot, with certainty, use as a control group animals that were immunized previous to the experimental ones, though that has been done by some investigators

TABLE I

The Effect of ACTH on the Concentration of Circulating Antibody When the Hormone Is Administered from the Onset of Immunization with Pneumococci

Mg antibody nitrogen per ml of serum at 14 and at 28 days after beginning immunization with pneumococci

After 14 days		After 28 days	
ACTH Group P	Control Group K	ACTH Group P	Control Group K
mg AbN/ml	*mg AbN/ml*	*mg AbN/ml*	*mg AbN/ml*
0.50	0.98	0.67	1.41
0.54	1.47	1.01	1.90
0.62	1.49	1.22	2.30
0.81	2.02	1.67	—
2.34	2.22	3.34	5.31
	2.30		4.72
	2.50		6.23
Mean 0.96	1.85	1.58	3.65

Five New Zealand red rabbits treated with ACTH approximately 0.5 to 1.0 mg Armour standard, every 8 hours for the entire period Seven control rabbits of same strain Reprinted by permission, from Bjørneboe, M Fischel, E E, and Stoerk, H C The effect of cortisone and adrenocorticotropic hormone on the concentration of circulating antibody *J Exper Med* 93, 37 (1951)

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shock, may be expected to be increased by adrenalectomy and decreased

ve were influenced by Dr. n connection with the ef- what might occur under physiologic conditions but the pharmacologic action of an excess of these hormones, just as an excess is required for the suppression of symptoms in various diseases. We certainly do not suppress them by fulfilling a need of the patient or by giving physiologic levels of hormone comparable to substitution therapy in Addison's disease

There are several types of studies on antibody production The obvious experiment is that in which there are two series of animals, both of them immunized simultaneously, but only one group treated with ACTH or cortisone Simultaneous control studies are necessary because, even with the most precise techniques, variables are probably present which cannot be appraised Therefore, we cannot, with certainty, use as a control group animals that were immunized previous to the experimental ones, though that has been done by some investigators

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Mean 0.96	1.85	1.58	3.65

Five New Zealand red rabbits treated with ACTH, approximately 0.5 to 1.0 mg Armour standard, every 8 hours for the entire period Seven control rabbits of same strain Reprinted, by permission, from BJØRNEBOE, M, FISCHEL, E E and STOERK, H C The effect of cortisone and adrenocorticotropic hormone on the concentration of circulating antibody *J Exper Med* 93, 37 (1951)

In the following experiment, done in collaboration with Dr. Bjorneboe and Dr Stoerk (5), several series of rabbits were immunized with a polyvalent pneumococcus vaccine, and were treated with either ACTH or cortisone. For the first small series, antibody levels fourteen and twenty-eight days after the beginning of immunization are shown in Table I ACTH, which was given in very small dosage, obviously introduced still another variable We felt guilty about arriving at a mean for only five animals, but there was enough of a difference to make us want to go on The median can perhaps be taken at .62 milligrams of antibody nitrogen in the first column, but in any case, there is a difference after fourteen days of immunization between the two groups The same animals were immunized for fourteen more days, with continued hormone administration to group P. The amount of antibody on the twenty-eighth day from each animal is arranged in columns 3 and 4 in the same order as in columns 1 and 2. Thus, it is seen that the first animal of column 1 had little arithmetical increase in circulating antibody (from 0.50 mg on the fourteenth day to 0 67 mg of antibody nitrogen per ml of serum on the twenty-eighth day). Some of the rabbits did show an appreciable increase. In toto, there was a difference in that the mean antibody content of the untreated group was twice that of the ACTH-treated group. Again, an apology is in order for the use of mean values, but to use median values would show even more pronounced differences.

With this group as an indicator, we went on to study two more series of animals Table II shows the amounts of antibody in two groups of rabbits given 10 mg and 2 5 mg of cortisone daily, the mean values of simultaneous control groups being indicated on the last line of the Table. The footnote to the Table lists the individual titers of the 38 control animals, 27 immunized simultaneously with the rabbits given 10 mg. of cortisone and 11 with the group receiving 2.5 mg

Some of the animals receiving 10 mg. cortisone daily died. Control animals also died, due to the toxicity of the large amounts of pneumococcus vaccine Early in the experiment, more of our cortisone animals died than did our control animals, which may cast some doubt on the findings. However, later, when we dealt with the effect of cortisone on antibody levels after the animal had been fully immunized, the cortisone-treated animals stood up much better than did the control animals, and the depression of antibody was again found. Illness, per se, did not seem to be directly responsible for the lowering of antibody levels, either in individual animals or according to group mortality figures.

Comparing the mean amounts of antibody after nine and fourteen days in the animals given 10 mg of cortisone with that of the corresponding control series, a striking difference is seen, but the difference

TABLE II

The Effect of Cortisone on the Concentration of Circulating Antibody When the Hormone Is Administered from the Onset of Immunization with Pneumococci

Mg antibody nitrogen per ml of serum

10 mg cortisone daily			2 5 mg cortisone daily	
Rabbit	9th day	14th day	Rabbit	14th day
	mg AbN/ml	*mg AbN/ml*		*mg AbN/ml*
B2	0 06	0 46	E24	0 49
B6	0 06	0 48	E8	0 62
B12	0 09		E29	0 67
B9	0 10	0 18	E20	0 70
B1	0 13		E28	0 80
B7	0 18	0 77	E26	0 85
B4	0 21		E23	0 85
			E5	0 85
			E27	1 06
			E7	1 19
Mean	0 12	0 55		0 81
Mean ±S E of groups without cortisone	0 27* ± 02	1 37* ± 13		1 17‡ ± 06

*0 27 and 1 47 are the average AbN/ml on the 9th and 14th days respectively of 27 rabbits of Groups A and C immunized simultaneously with the B group animals Individual titers of these controls on the 9th and 14th days are as follows 0 13, 0 49, 0 28, 0 54, 0 19, 0 60, 0 21, 0 61, 0 07 0 69, 0 31, 0 75, 0 22, 0 99, 0 32, 1 12 0 23 1 15, 0 19, 1 20, 0 16, 1 26, 0 56, 1 55, 0 28, 1 54, 0 27, 1 34, 0 40, 1 54, 0 22, 1 57, 0 37, 1 61, 0 12, 1 68, 0 35, 1 68, 0 22, 1 95, 0 17, 2 16, 0 16, 2 23, 0 27, 2 34, 0 52, 2 40, 0 43 2 52, 0 31, 3 05, 0 47 (9th day only)

‡1 17 is the average antibody nitrogen level per ml on the 14th day of 11 untreated control animals of Group F immunized simultaneously with the E group animals These controls had the following titers 0 41, 0 52, 0 71, 0 85, 1 04 1 11, 1 19, 1 34, 1 46, 2 04, 2 18

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is probably not marked enough to show up in immunological techniques which employ the traditional, and unreliable, double dilution methods A one tube or two tube difference may not, perhaps, have been detected in this series of animals, or, conversely, a difference of several tubes might have resulted

When the amount of cortisone was reduced to 2 5 mg daily, there

was a higher level of antibody at the fourteenth day. This experiment, shown in Table II, was conducted about two or three months later, and in this instance we ran eleven untreated controls. As can be seen, the control group yielded a somewhat lower mean value on the fourteenth day than did the control group previously employed, which is why simultaneous controls must be run The value is within the range but not quite that which was obtained previously

What happens to antibody levels if cortisone is administered after immunization has become well established The control group in Table II, after fourteen days of immunization, could be divided into two groups, A and C Figure 47 shows what happened when the rabbits of group A were given cortisone, 5 mg. for the first four days and then 2 5 mg for three days. The mean serum level at the onset of cortisone administration to this group of 11 rabbits was 1.79 mg of antibody nitrogen per ml

Ragan: Are you immunizing these rabbits with one injection at the beginning?

Fischel No, these are repeated injections.

Ragan: Repeated even when they are on cortisone?

Fischel. Continued both in the control and the cortisone groups We used Bjørneboe's method of immunization, which results in rather high antibody levels

Lillie: What is your determination method?

Fischel: The quantitative agglutination method of Heidelberger and Kabat (6). Whole pneumococci are agglutinated with the antibody and washed The supernate must be tested to see that all the antibody has been removed After repeated washings with cold saline, the antibody and pneumococci are analyzed for nitrogen content, using appropriate controls to determine the nitrogen in the pneumococcus vaccine itself All these determinations are done in duplicate.

To return to Figure 47, after one week of further immunization, the untreated group had a rise of antibody nitrogen to 162 per cent of its mean level at fourteen days, as indicated by the dotted line. The group receiving cortisone had a slight drop to 88 per cent of their fourteen day level, which may not be a significant drop. Essentially, the mean value remained about the same, while it rose in the untreated group C Note the wide variation within the groups, as has been well described in the literature One or two animals had increases for two or three days following cortisone, but they leveled off. This experiment was repeated at another time, a control group of animals received no cortisone and had an increase in mean antibody content from 1.85 to 3 65 mg AbN per ml in fourteen days The group F animals shown in Figure 48 had a definite depression after fourteen days of cortisone

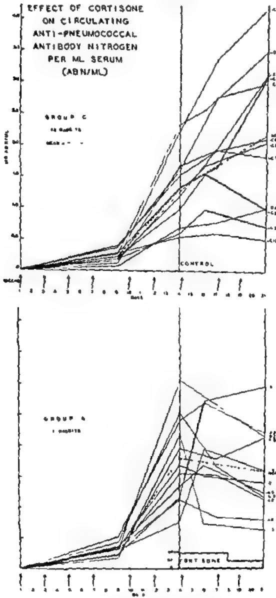

FIGURE 47 Reprinted, by permission, from BJORNEBOE, M, FISCHEL, E E., and STOERK, H C The effect of cortisone and adrenocorticotropic hormone on the concentration of circulating antibody *J Exper Med* 93, 37 (1951)

Dempsey How much is known as to the explanation for such great variability in what is, presumably, a standard population of animals to begin with? Is there any physiologic variant or any known mechanism that can account for this? Is there any way of getting a more standard animal for this kind of experiment?

Fischel: The problem is well recognized for almost every species in which antibody has been determined. It has been extensively studied in rabbits and horses Even humans have been divided into 10 per cent very poor antibody producers, 10 per cent extremely good, and the rest forming a nice bell-shaped curve

Dempsey: For example, you are showing here, as I understand it, that cortisone depresses the level of antibody production The more cortisone given, the less the level of antibody is at any time. Now, starting out with a group of rabbits, the normal level of adrenal activity might vary a great deal Some of them might have minor infections, others might not, some might have cold feet and others might not So I question your statement that adrenalectomized animals cannot be used in this type of experiment Might it not be better deliberately to use adrenalectomized animals, maintained on a known dose of cortisone At least the endocrine baseline would be known in that case.

Fischel: It is a very good point. When I was talking about adrenalectomized animals, I meant comparing adrenalectomized animals and so-called normal animals given substitution therapy but not made hyperadrenal

Dempsey: There is a fluctuating endocrine baseline here, and, in any kind of animal experiment of this kind, there is a varying genetic baseline

Fischel: I doubt if the variation in antibody production is completely ascribable to an adrenal difference The differences between the animals that are very good antibody producers despite cortisone administration and the others with very low antibody levels during administration of the same dosage of cortisone are much more than we can attribute to variations in endogenous cortical hormone.

Mirsky: Actually, such large variances are seen in other situations related to adrenal cortical function For example, the synthesis of glycogen in brown fat of the rat shows marked variability when animals are chosen at random. On the other hand, as Dempsey just suggested and as has been done by Engel (7), if the animal is adrenalectomized but maintained with some specific dose of hormone, the variability is reduced More significant is the fact demonstrated by Engel that when rats are exposed to the cold and treated as mentioned, then the action of insulin on glycogen formation in the fat is markedly enhanced The point I am trying to make is that by maintaining adrenal-

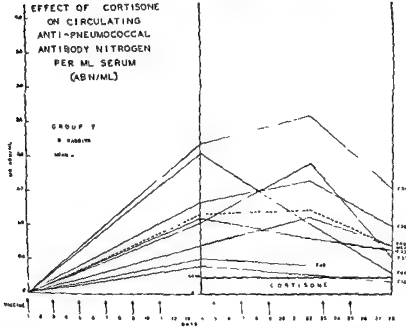

FIGURE 48 Reprinted, by permission, from BJORNEBOE, M, FISCHEL, E E, and STOERK, H C The effect of cortisone and adrenocorticotropic hormone on the concentration of circulating antibody *J Exper Med* 93, 37 (1951)

ectomized animals with exogenous hormone or by stimulating their own adrenals, a more constant endocrine baseline is achieved and the spread is diminished

Dempsey There is another endocrine phenomenon that can give this great variation For example, a completely myxedematous patient responds to a very small amount of thyroxin or thyroid hormone with a great increase in metabolism However, in an euthyroid individual, it takes a rather large dose to budge his metabolism, and if it is a hyperthyroid individual to begin with, then his metabolism practically cannot be changed with any dose of exogenous hormone Thus, depending upon the baseline of endogenous hormone, the effect of administered doses is very different

Fischel. The analogy is intriguing, but the experience with cortisone is perhaps contrary to the experience with thyroid Patients with adrenogenital syndrome do not require huge excesses of cortisone to suppress adrenal activity

Ragan: But the suppression probably is derived from a different type of adrenal activity.

Dempsey: Yes, it is mixed

Fischel: As a corollary, Addisonian patients do well on less cortisone than is used in the treatment of rheumatoid arthritis, but they do require an optimal dosage. They cannot be maintained on fractions of that optimal dosage, the way a myxedematous patient may be on fractions of his thyroid requirement.

Gyorgy: The curves presented in Figure 48 are in favor of this thyroid analogy because the two lowest remain the lowest, even after cortisone.

Dempsey: That's right; that is what suggested it to me.

Robb-Smith: I think Hartley has shown that far greater standardization of the immune response is obtained if a pure strain of animal is used and also if the diet is very carefully controlled. There is no doubt that the dietary factor does make a great difference to the response.

Lillie: Another factor which might be very important is that the higher antibody responses may correlate with the presence of intercurrent infections in the animals, the phenomenon of increased immune response because of other infections

Fischel We have tried to keep an eye out for that type of thing We find, at autopsy, that some animals do have cysts or parasitic infestations of one kind or another, but there does not seem to be a correlation with a low or high antibody level. In this connection, it might be well not to try to pin the variation down to one trait of the animal. As an illustration of how variable these things can be, Dr. Heidelberger has immunized people with two different pneumococcus polysaccharides, and occasionally an individual will show a high antibody response to one of the polysaccharides and a very low response to another (8) Thus, there is a complete dissociation in the same individual to two antigens which are almost alike in composition. Larson and Tomlinson (9) also have found the same dissociation of response pattern to two pneumococcus polysaccharides in the same individual.

Dempsey: In other words, there are many variables we do not know about.

György: It is an important point with inbred rabbits.

Fischel: Heredity certainly plays an important part in antibody production, as shown in the experiments that Lurie and others have done, but it is difficult to think of heredity as applying to the antibody-producing mechanism as a whole since reactions to related, but chemically distinct, antigens may vary extremely in the same person. Perhaps you would like to know the histologic findings and then we can discuss some of the other studies. The normal spleen follicle from rabbits who

were in the animal room at the same time and came from the same dealer but who were not immunized is not very remarkable. Immunization, as has been described by several authors, causes the appearance of a large, pale germinal center in the spleen follicle. Dr Bjørneboe found a rough correlation between spleen weight and amount of antibody production some years ago, and this was again found by us. In the animal given cortisone, the architecture of the follicle is destroyed (5). The destruction extends to many cell types, not alone lymphocytes but also plasma cells, and I suppose to fibroblasts if we could pin them down as such. There is complete chaos, which is in keeping with the diminution in the size of all the lymphoid tissues, spleen and others, following an increase in adrenal cortical activity. These findings are in contradiction to the data of Chase, White, and Dougherty (10) which show an increase in antibody production during adrenal cortical hormone administration. However, the techniques by which the antibody analyses were done left much to be desired in that the investigators used heterogenous antigen and double dilution determinations at different times. Animals were killed periodically and the serum from a few animals was pooled for the antibody determination, but, with the wide variability in individual titers, it is dangerous to draw conclusions from pooled serums. One of these animals could raise the titer of the pooled serums considerably above what might have been present in the others, or contrariwise.

György: Did the investigators say why cortisone increased production of antibody? Did they not claim that lymphocytes are affected by cortisone and the antibodies of the lymphocytes are liberated?

Fischel: There are two experiments, or more than two, and we are coming to the one on the anamnestic reaction in a moment. That is an allied *problem. I think their interpretation has been altered somewhat* since then.

Drs Eisen, Stoerk, and others (11) tried to repeat the experiment of Chase, Dougherty, and White by giving adrenal cortical hormone during immunization, using somewhat the same system as well as a quantitative immunochemical system, and they obtained no evidence of increased production. Germuth and Ottinger (12, 13) have published a similar series of experiments, using the quantitative precipitin technique and crystalline egg albumin as the antigen. Their results and ours agree completely as far as suppression of antibody goes. Because we were working at a much higher level of antibody production, the inhibition of these levels by cortisone in our study may have been a little more pronounced in proportion. The findings are now quite well established, when purified systems are employed. In humans, there has been a rash of papers on the effect of cortisone on antibody

and, again, we have the problem that most investigators deal with agglutination of typhoid organisms, or equally suspect techniques, where changes of as much as 1000 per cent may be expected due to technique alone, especially when tests are not done simultaneously. Interpretation of results, therefore, is very difficult

Mirick (14) has immunized humans with pneumococcus polysaccharide, but, unfortunately, the antibody was measured by the mouse protection technique which employs double dilution of antiserum. He achieved no great change before and after cortisone and attributed much of the finding to species difference, feeling that man did not react like the rabbits studied by Germuth and Ottinger and by our own group. However, not only was the antibody measuring technique a double dilution one, which may account for much of the difference, but immunization with pneumococcus polysaccharide results in a type of immunity which immunologists have heretofore not been familiar with experimentally, although probably it has been known to exist in the form of measles immunity and other examples of prolonged immunity When a human is immunized with pneumococcus polysaccharide, within two to four weeks he achieves a level of antibody production or of antibody circulating in the serum which persists essentially unchanged for years. It does not fall off the way antibodies usually do after a certain time, nor does it rise further when booster shots are given several years later. Drs Heidelberger, MacLeod, and others (8, 15) have shown that. We are, therefore, dealing with an antigen which, as these latter investigators suggest, probably persists in the body for years

Porter Has the antigen been demonstrated in the circulation?

Fischel: I do not know. It does not seem likely that it would be in the circulation with antibody there also. Their guess is that the antigen probably persists in cells for years. At any rate, further injections of antigen do not act like a renewed exposure. It is just taking coals to Newcastle They already have their full antibody titer.

Larson (9) has immunized patients with rheumatoid arthritis, some receiving cortisone and some not receiving cortisone, with pneumococcus polysaccharide, and, using the quantitative precipitin technique, found no great difference He has about a dozen patients in each group.

Havens (16) has used diphtheria toxoid and, again, found no great change due to ACTH Unfortunately, in his experiment, ACTH was given after the immunizing dose, and I do not believe it can be said that the animal or the human was hyperadrenal at the time the antigen was given. By analogy with x-ray studies, irradiation after exposure to the antigen does not seem to have as much of an effect as if given immediately before immunization

Lillie: In regard to the persistence of bacterial polysaccharides, an

experience of mine might be of interest Sometime back, after periodic had been introduced, we were studying lymph follicles in the intestine of rabbits We saw a good deal of bacterial invasion there, and close to the surface we saw bacteria which could be demonstrated with Achard's test. They retained their ribonucleic acid Farther in, we found bacteria which had lost their capacity to stain with basic anilin dyes, but still retained their Gram stain Still farther were organisms which had lost both the ribonucleic staining and the Gram staining The polysaccharide of their capsules however, as demonstrated by the periodic test, was still present in the phagocytes in such form that many of the bacteria were well preserved This might indicate that an antigenic fraction of these particular Gram-positive organisms might well have stayed around for a considerably longer time than the microscopically recognizable organism That may cast a bit of light on the idea of storage of polysaccharides

Fischel: As I think back to Dr. Porter's question and your comment, I believe there have been studies to show that mice, given too much pneumococcus polysaccharide, never form antibody because the antigen does persist, the mouse, in this respect, acts somewhat like the human The rabbit does not become immunized by exposure to the polysaccharide alone.

Meyer: How long do you think that persists?

Fischel. I don't know, but I recall that Dr Harry Rose told me about one mouse that had at some time been injected with a type I pneumococcus and, having received penicillin, did not die The mouse was used some weeks later for an intraperitioneal injection of some totally unrelated material, whereupon the mouse got sick and died of a pneumococcus peritonitis due to the type I organism This was persistence of live bacteria in the tissue for over a month

To return to our point of departure, in humans, using this peculiar antigen stimulus, pneumococcus polysaccharide, Larson found no difference in rheumatoid patients with and without cortisone However, he has allowed me to say that his current studies are showing a decided difference, due to cortisone, in patients with leukemia With acute leukemia, he has found a very marked increase in production of antibodies He is prepared to report soon, I believe

György: What type of leukemia, myelogenous or lymphatic?

Fischel. Certain acute leukemias give the marked increase In acute leukemias, it is difficult to tell what cell type is predominant, but he obtains phenomenal antibody production in some Others act like normal individuals and he supposes they might have lymphatic leukemia Chronic myelogenous leukemias exhibit somewhat elevated antibody production compared with normal values, but chronic lymphatic leu-

kemias have practically no antibody formation The increased production in acute leukemia is brought down, when the patients are treated with cortisone, to levels comparable with those of other patients There we have a factor, perhaps, of lymphoid tissue, and by lymphoid tissue, I do not mean just lymphocytes but the whole gamut of cells that are present in lymphoid tissue and which probably contribute to the excessive amount of antibody production

Gyorgy. You have to include plasma cells, I suppose

Fischel. Oh, yes I have no intimate knowledge of cell physiology, but, while my sympathies are very much in favor of plasma cells contributing to circulating antibody, I still think that almost every cell retains some properties of reacting to noxious agents and, as such, may be sensitized, although they may not contribute to plasma antibody. There are many sensitivity reactions which suggest that

Lillie It is entirely possible that the leukemic lymphocyte has lost that capacity in the course of its neoplasia

Gyorgy Yes, it is too immature, undifferentiated

Lillie: Yes, if it is a normal function of the lymphocyte

György But the lymphocytes are not normal any more.

Fischel Either that, Dr Lillie, or perhaps the function may be enhanced by illness, as Larson's studies for myelogenous leukemia show. Certainly the plasma cell myeloma is characterized by increases in protein production

Robb-Smith· But not ordinary response to antibody I thought there was good evidence in leukemia, leaving out cortisone, that there is no enhanced antibody response to a fairly large range of antigens. Is it that these leukemic patients who were not under cortisone had an enhanced response, and when they received cortisone, they had a normal response?

Fischel. Exactly With cortisone, the antibody level comes down to a normal range

Another aspect of antibody production is the so-called anamnestic response or secondary response which has been widely studied, particularly in connection with booster shots of tetanus, typhoid, or diphtheria. The literature is replete with comments about the protection that certain stress situations give to infections, for example, that typhus fever may be accompanied by a nonspecific rise in typhoid antibodies Topley and Wilson (17) have well stated the point that such studies really do not exclude the possibility that similar chemical groupings are involved in stimulating antibody, if such is stimulated I think most people in the field today feel very much in accord with Landsteiner's (18) view of the specificity of serologic reactions, that there is no such thing as an anamnestic response to nonspecific stimuli However, this hypoth-

esis was examined by Dougherty and White (19) and they felt that stress situations may cause an outpouring of adrenocortical hormones, which may result in the lysis of lymphocytes and an increase of antibody in the circulation We examined this in 1947 with Drs LeMay and Kabat (20), using the quantitative immunochemical procedure to measure change in precipitins following the administration of ACTH, and we could not confirm this Drs Eisen, Stoerk, and others (11) had previously done a similar type of experiment, with equally unimpressive results

Table III shows the results of a study of six rabbits Six others were treated with x-ray with similar outcome After immunization, the rabbits had the amount of circulating antibody listed in the first column under August 1947, and expressed as micrograms of antibody nitrogen per milliliter Again, there is quite a little variation. They were allowed to rest for three months, as had been done in the previously cited work (20), and the antibody in the circulation fell as indicated. They were given ACTH and followed serially at six, twelve, and forty-eight hours after ACTH, and in no case was there a rise, although lymphocytes were depressed, as might be expected Indeed, in some of the animals there seemed to be a slight drop in circulating antibody concentration At the time we did not feel that it was a significant drop and did not pursue this In view of our more recent work on depression of antibody after immunization is established, the slight drop noted previously may have been of significance

Dr de Vries (21) in Montreal has done a similar quantitative immunochemical study, but without waiting a full three months for antibody titers to fall Therefore, the experiment started with a little higher antibody level, and more impressive falls in antibody titer resulted after giving ACTH The results of her studies and ours were directly contrary to those reported by Dougherty, Chase, and White, although all of us recorded the expected fall in circulating lymphocytes In two humans, Larson and Tomlinson (9) gave cortisone after immunization to pneumococcus polysaccharide was well established, and, although it was not felt that there was a significant difference, nevertheless the figures show a drop in antibody to two different penumococcus types in each of the patients who received cortisone The drop was consistent and more marked in all four antibody levels than occurred in several other patients who did not receive cortisone two or four weeks after immunization

From the problems of active immunization or anamnestic response, we can go on to the study of the events which occur when antibody is given passively to an animal, which becomes a problem of protein metabolism This will also be of interest later because the sensitivity reactions were induced by passive immunization

TABLE III

The Effect of Adrenocorticotropic Hormone on the Antibody Content of Serum, the Amount of Total White Cells and of Lymphocytes. 25 mg Adrenocorticotropic Hormone Given on Nov. 13th.

Rabbit		August 1947	November 11	November 13 6 hrs after stimulus	November 13 12 hrs after stimulus	48 hrs after stimulus
C3	WBC		13,250	16,250	12,150	7,700
	Lymph		9,500	2,700	4,130	5,080
	μg AbN/ml	170	2	0	0	0
S1	WBC		10,700	3,600		
	Lymph		6,250	685		
	μg AbN/ml	180	10	3		
S3	WBC		10,550	15,000	5,190	
	Lymph		5,180	2,550	1,500	
	μg AbN/ml	1,060	28	20	15	
SM	WBC		13,350	10,450	7,900	9,700
	Lymph		7,480	3,350	3,550	5,920
	μg AbN/ml	320	18	11	12	11
X6	WBC		28,650	17,600	28,600	12,000
	Lymph		11,750	5,450	12,600	7,550
	μg AbN/ml	210	12	10	10	10
N2	WBC		11,600	11,700	13,900	4,300
	Lymph		8,020	1,650	3,600	1,115
	μg AbN/ml	63	0	0	0	0

Reprinted, by permission, from FISCHEL, E E, LEMAY, M, *and* KABAT, E A *The effect of adrenocorticotropic hormone and x-ray on the amount of circulating antibody* J Immunol **61**, 89 (1949)

In our active immunization experiments, the question arose: Is antibody being decreased by hormone because of augmented catabolism of the protein, or is there a lack of synthesis of the antibody globulin? We adopted a technique which Heidelberger, Rittenberg, and their co-workers (22) had used previously ... ance rate
of tagged antibody Bjørneboe (23)
persistence of globulin in the circul...
antipneumococcal rabbit serum intravenously The animal was then bled at intervals and the amount of residual antibody determined with

antigen, a fairly convenient technique with which to follow the disappearance rate of the protein.

We fractionated pooled rabbit antipneumococcus serum by salt fractionation to obtain the globulin and found that we had enough antibody to inject four rabbits. Two of the rabbits were pretreated with cortisone and cortisone was continuously administered for a period of ten days (24) In Figure 49, the dotted line represents the disappearance rate of the antibody from the circulation of the control rabbits, B and F, and the straight line is the disappearance rate from the cortisone-treated animals The approximation of these curves is very good

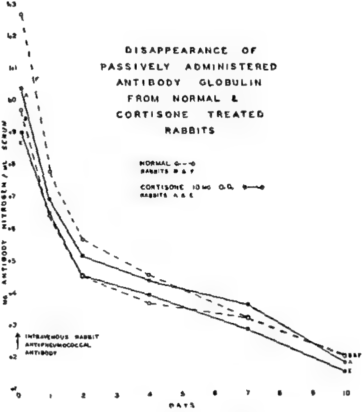

FIGURE 49 Reprinted, by permission, from FISCHEL, E E, STOERK, H C and BJORNEBOE, M Failure of cortisone to affect rate of disappearance of antibody protein *Proc Soc Exper Biol & Med* 77, 111 (1951)

for a biologic experiment There is no difference in the disappearance of passively administered antibody from the circulation of control and cortisone-treated animals. As an aside, it is striking that these curves can be superimposed on those of the four rabbits studied previously by this method by two independent groups, Heidelberger and his co-workers in New York in 1942 (22), and Bjørneboe in Copenhagen in 1945 (23).

There are similar slopes in all eight rabbits studied, with the characteristic change in slope on the second day. While it is perhaps not too accurate to do so, if half-lives are calculated there is a half-life for passive antibody of about five days from the latter part of the curve, the essentially straight-line disappearance curve Therefore, with some qualification, I think it can be concluded that cortisone does not increase the breakdown of antibody globulin, the qualification being that since these rabbits had not synthesized this particular rabbit globulin, they may not perhaps have had the apparatus to break it down readily. However, there are very beautiful studies with C^{14} glycine and other materials, showing that cortisone actually inhibits the incorporation of labelled amino acid into protein (25). Our current feeling, therefore, is that the low antibody levels obtained in our active immunization experiment may be ascribed to an interference with the synthesis of globulin by cortisone From the histologic level, the sections on spleen follicles add further confirmation to that hypothesis, namely, that the tissues usually associated with antibody production certainly have been very much disturbed morphologically by cortisone administration (5).

In man, administering antibody passively is an established diagnostic technique in allergy, the so-called Prausnitz-Kustner reaction, and Stollerman and his co-workers (26) have found that this reaction is not altered by cortisone administration There have been two other reports confirming these findings Dr. Holbrook, do you think that the increased amino aciduria following ACTH or cortisone is due to a breakdown of protein or a lack of incorporation?

Holbrook I do not know the answer. We have tried to study this and the assumption from the evidence is that it is probably due to lack of synthesis. But it is something more than that. We do not have the answer to a lot of things that happen with the increased amino acid outpouring on the administration of ACTH and cortisone. However, I am reasonably sure that lack of synthesis plays a role in it

Fischel: From our work, we can only say that the catabolism of this particular globulin is not accelerated, but it is possible that breakdown of tissue occurs in other sites.

Holbrook: That is right.

Fischel We are currently obtaining a further indication that the syn-

thesis of antibody is inhibited by cortisone by studying the specific anamnestic or secondary response When a previously immunized animal is re-exposed to specific antigen, a very rapid rate of antibody production is usually found The magnitude of the response in the first week is so great that the rate of destruction of protein may be considered negligible. Preliminary results indicate that cortisone may inhibit this response which, by and large, is one of synthesis of protein

There are sufficient data to indicate that allergic reactions are greatly dependent on the amount of antibody present for reaction (4) Having discussed the effect of hormone on antibody, suppose we go into several of the allergic reactions Leger, Leith, and Rose (27) produced anaphylaxis in two groups of animals One group received ACTH with no effect on the severity of anaphylactic shock In their experiments, active immunization with horse serum was used, which can be unfortunate but, in this instance, their groups were comparable in the results obtained, both as regards mortality and the incidence of milder reactions There was approximately fifty per cent mortality in each group which showed that they were probably dealing with amounts of antibodies that were threshold Experiments in which anaphylaxis is produced by a high degree of immunization are not valid in attempts at protection because it is difficult to expect a pharmaceutic agent to prevent the effect of an extremely potent stimulus

We have done the experiment on anaphylaxis using amounts of antiovalbumin antibody known to be about the minimal amount which would result in anaphylactic death in guinea pigs (28) This amount has been found to vary with the duration of the latent period before administration of the shocking dose (29) A series of animals given varying dosages of ACTH and cortisone, as shown in Table IV, were shocked, together with untreated controls Since the results in each small group are comparable, they are summarized at the bottom for facility in appraisal Some of the guinea pigs were 300 to 400, rather than 250, grams in weight Therefore, a greater than usual number of the animals survived the shock in both control and treated groups Of the fourteen animals given hormone, four died, comparable to the four untreated animals that died, and so on down the line, with no variations due to hormone administration In retrospect, these doses of hormone may have been too little and we are repeating these studies with Dr. Carl Nelson, using very large quantities of hormone Thus far, giving as much as 25 mg of cortisone intramuscularly for three days to 250 gram guinea pigs, we have found no appreciable protection when the guinea pigs were sensitized with 25 μg of antibody nitrogen, but some slight protection was obtained when the sensitizing dose was half that amount Of course, these are huge quantities of steroid to inject into

TABLE IV

Lack of Effect of Adrenal Cortical Hormones on Anaphylaxis Induced in Guinea Pigs by Passive Sensitization

Sensitizing dose of antibody nitrogen — I V	Latent period and shocking dose — I.V	Treated Group		Untreated Group
		Hormone given before shock	*Severity of Shock*	*Severity of shock*
240 μg	1 hr 0 16 mg Ea N	2 mg ACTH 8 hrs. before	++++ ++ ++ ++	++++ ++++ +++ +++
30 μg	48 hrs 0 16 mg Ea N	2 mg. ACTH q 6 h for 2 days	++++ ++++ ++++ +++ +++	++++ ++++ ++ +
32 μg	48 hrs 0 06 mg Ea N (gp 300 400 Gm)	Cortisone 2.5 mg I.M. for 3 days	+++ +++ +++ ++ ++	+++ +++ ++ ++ +
Number of animals with		++++ (death)	4	4
		+++ (severe)	5	4
		++ (moderate)	5	3
		+ (mild)	0	2
		Total	14	13

so small an animal, and further studies are necessary to control nonspecific factors, so many of which have been known to inhibit anaphylaxis in the past I think it is proper to say that in the guinea pig, our studies, those of Leger, *et al.* (27), of Stoerk (30), and of Grabar, *et al.* (31) all indicate that cortisone or ACTH exerts no appreciable protection against anaphylactic shock despite the transient suppression of analogous symptoms in humans clinically.

In mice, another situation seems to obtain, although it is fairly difficult to evaluate Mouse anaphylaxis has not been studied quantitatively as has guinea pig anaphylaxis, partly because of the small blood volume In two sets of experiments, one by Dougherty (32) and one by Nelson, Fox, and Freeman (33), cortisone seems to inhibit anaphylactic death. Dougherty's work included adrenalectomized mice, which,

like other adrenalectomized animals, may be more susceptible to many procedures and may be made more resistant by replacement therapy Also, he judged the effect by the amount of horse serum needed to shock, which is a much less reliable indication than is the amount of antibody (4) The amount of cortisone is, obviously, very much related to the degree of inhibition of anaphylaxis, and, in Nelson's experiment, almost complete inhibition obtained with 3 mg. of cortisone to a 20 gram mouse, which, I think it will be agreed, is quote a lot When the dosage was dropped to 1 5 or 0 75, much less protection was afforded.

Ragan: How much cortisone were the guinea pigs given?

Fischel. Cortisone was given in 2 5 mg intramuscularly for three days, which is very small for the guinea pig

Ragan: The guinea pig is about the most resistant of all animals to the effect of cortisone.

Fischel: We have since found that out in another connection and that is why we are currently giving such huge quantities as 25 mg daily

Ragan: Wolbach's (34) recent paper indicates that when one discusses the effects of cortisone on a reaction, it is important to consider species differences and variations in dosage

Fischel: And the dosage is not on a weight basis?

Ragan The dosage is not on a weight basis at all In the effect of cortisone on wound repair, the mouse, guinea pig, and rat are the most resistant, then the dog, the rabbit, and finally the human

Travell. Which species would you say is the most similar to man in that respect?

Ragan The rabbit is most similar to man in relation to this particular phase. There is one very definite difference, which has been shown by Findlay and Howes (35), that if a rabbit which is being given cortisone is starved, an effect can be produced which is not producible in a fed rabbit with the same dose of cortisone

Fischel: The independence of weight to dosage has also been shown in the 2 kg monkey Kabat, Wolf, and Bezer (36) inhibited allergic encephalomyelitis in monkeys with 40 mg of cortisone, which is a sizable dose in human terms In mice, with only 0 1 mg of cortisone, the protection to tuberculosis that is given by previous inoculation with dead tubercle bacilli is completely inhibited, or almost completely inhibited, as shown by Solotorovsky, Gregory, and Stoerk (37) Therefore, this dose of cortisone produces some hyperadrenal effect in the mouse, but it requires thirty times that to inhibit the anaphylactic reaction in the mouse (33)

Ragan: In the mouse, a dose of 2 mg a day to a 20 gram mouse will, during the period of time observed, suppress the repair processes,

whereas with 1 mg a day, the suppression is extremely variable

Fischel: That is further evidence of what I wish to point out, that these suppressive effects on tissue repair or, in this case, on anaphylaxis, require doses of cortisone which are many times that which is necessary for other hyperadrenal effects, such as inhibition of the protection endowed by vaccination with dead tubercle bacilli This phenomenon obtains with many pharmaceutic agents; that is, certain systems are more readily affected by lower dosage and therefore can perhaps be thought of as being more pertinent to the study of the action of a drug.

Holbrook: Do you believe it is a question of the amount of the drug that it takes to produce a certain degree of hyperadrenalism, or do you think there is another factor that has to do with dosage?

Fischel: Most of the things we have been talking about involve a degree of hyperadrenalism, but the answer to your question would appear to depend on the system used. If eosinophil drop is being studied, a very small dose of hormone may affect it, but that dose will produce no clinical effect on an inflammatory reaction Certain patients with widespread and rather serious diseases, such as lupus, seem to require much more hormone for suppression of manifestations of the disease than do patients with milder diseases

Holbrook: Yes, but that has nothing to do with the degree of hyperadrenalism that I am referring to.

Fischel I don't know I think it may involve the degree of destruction by the disease or the degree of utilization of the hormone in the inflammatory site, any excess contributing to other manifestations of hyperadrenalism elsewhere.

Meyer: Wouldn't you think that the reactivity to cortisone or ACTH of the mesenchymal or other cells differs among different types of cells The term "hyperadrenalism" would denote the overall effect on all cells and organs, whereas in your experiments, as in wound healing, you are dealing with a more localized cellular effect?

Fischel: Hasn't Jailer (38) some evidence that cortisone is utilized *in situ* in inflammatory sites? At a cellular level at any rate, cortisone is inactivated But I think that the dosage has to be considered in relation to how much may be utilized. If it can be conceived, hypothetically of course, that actively metabolizing inflammatory tissue is breaking down or utilizing cortisone, more cortisone may be needed to have some excess to produce a so-called hyperadrenal effect, the clinical signs of puffy face, or what you will.

Ragan: Until we have some concept of what actually is going on, hyperadrenalism can be used as a generic term For example, one may talk about an area of hyperadrenalism as produced when the subconjunctiva is injected, but that is just a euphemism until it is actually

known what we are doing. I don't think it is necessary to consider a particular group of symptoms, except as they are manifestations of [illegible] bout [illegible] may [illegible] ent, [illegible] the

Fischel: But it is a good descriptive name for a similar situation which we associate with excess adrenocortical hormone

Ragan: Yes, we associate it with excess adrenocortical hormone except that in one situation the adrenal is enlarged In this situation, following cortisone administration there is always a small adrenal

Fischel: As would be expected if cortisone were given, but not if ACTH were given

Ragan: That's right, but, even with ACTH, there is probably a suppression of ACTH production by the pituitary

Fischel: The results, or rather the lack of results, with cortisone and ACTH on anaphylaxis have been presented

If antibody is injected passively into a local skin site and a half hour later antigen is given intravenously, in a few hours signs of inflammation result and persist for twenty-four hours or so, with different degrees of erythema, edema, induration, and central blanching or necrosis being produced, the severity depending again on the amount of antibody present, and less so on the amount of antigen, over a threshold level obviously (39).

Varying amounts of ACTH were administered to the rabbits, as noted in Table V. The severity of the Arthus reactions in the treated groups are comparable, percentagewise, to those in the corresponding control groups Thus, in rabbits receiving 0 2 mg antibody nitrogen, nine of ten controls gave a four plus reaction, and fourteen of the sixteen animals receiving various dosages of ACTH had comparable reactions, and so on down the line Arthus reactions may also be induced in guinea pigs by quantitative immunochemical methods (40), and our results with ACTH treatment are also shown in Table V In some instances, the pneumococcus immunized rabbits were given rabbit anti-ovalbumin and then crystalline egg albumin to elicit an Arthus reaction at a time when they were quite hyperadrenal from fourteen days of ACTH treatment; the Arthus reactions, again, did not vary from those elicited in a comparable group of animals not given ACTH Those results, although in animals receiving ACTH for a long period of time, are not included in Table V because the control and treated animals both were being immunized with pneumococci and therefore differed in some respects from the animals in Table V However, compared with

TABLE V

Lack of Effect of ACTH on the Arthus Reaction Passively Induced With Known Amounts of Anti-ovalbumin Nitrogen (AbN) and Crystalline Egg Albumin

Rabbits

	No tests	0.2 mg AbN ++++	+++	No tests	++	0.02 mg AbN +	±	0
Control	10	9	1	10	1	6	1	2
ACTH Total	16	14	2	16		12	3	1
2 mg / day × 5 days	6	4	2	6		5		1
6 mg 6 hrs before	6	6		6		5	1	
8 mg /Kilo/day in 4 doses	4	4		4		2	2	

Guinea Pigs

	No tests	0.2 mg AbN ++++	+++	No tests	+++	0.01 mg AbN ++	+	0
Control	6	5	1	6		3	3	
ACTH Total	11	10	1	11	1	7	2	1
1 mg /day × 5 days	4	4		4		2	1	1
3 mg 6 hrs before	4	4		4	1	2	1	
32 mg /Kilo/day in 4 doses	3	2	1	3		3		

Reprinted, by permission, from Fischel, E. E.: Relationship of adrenal cortical activity to immune responses. *Bull New York Acad Med* 26, 255 (1950)

each other, these animals showed no differences in Arthus reactions, although the amount of hormone given was enough to diminish antibody production to pneumococci.

Holbrook: Dr Fischel, is it your conclusion that, once the antibody is present, the reaction between antigen and antibody is not altered with the addition of ACTH or cortisone?

Fischel: That is correct. This reaction, whether it is at the threshold level, that is, minimum one plus or our so-called maximum, although we can have a much greater maximum if we give more antibody, is not

altered. It is not, therefore, a question of having too severe a reaction that cannot possibly be altered

Again, we come to the problem of dosage level of hormone However, other substances and conditions have been found to inhibit the Arthus reaction which are not as potent as cortisone in some respects Rabbits occasionally have an ingrowing of the incisors into the maxilla so that they can eat only a very small quantity of hay or rabbit food; such rabbits lose weight and are in a state of semi-starvation They will stay alive for some period of time Any skin reaction, such as the passive Arthus reaction, cannot be elicited Therefore, skin reactivity plays a big part in this type of study Although we have not tried it, perhaps a v[illegible]

that level.

inhibit the

vaccine also inhibit development of the passive Arthus reaction But salicylates, cortisone or ACTH, and pyribenzamine are among the agents which we have tried without any appreciable effect Some inhibition of the early stages of inflammation was found with salicylate (42)

There are many publications on the active Arthus reaction but, as I pointed out, they involve active antibody production and so many varia-[illegible]

reaction because antibody levels were also depressed But he very well confirmed our finding that, given a known amount of antibody passively, cortisone-treated animals did not have any different reaction from control animals, that is, the antigen-antibody reaction per se is not inhibited.

The reason why we belabor the Arthus reaction is that it is a type or a pattern for the occurrence of allergic reactions in many parts of the body Experimentally, serum sickness, nephrotoxic nephritis and other antigen-antibody reactions have been studied, chiefly with no effect from cortisone or ACTH. In the case of nephrotoxic nephritis, for example, where a known amount of anti-kidney serum is injected into an animal, Drs. Knowlton, *et al.* (43) and Hackel, *et al* (44), have shown that cortisone has no inhibiting effect on the resulting nephritis However, in serum disease which is actively induced in animals, such as the experiments reported by Rich (45) or Seifter (46) and their respective co-workers, horse serum arteritis, nephritis, and carditis are apparently inhibited I think this is in large part due not only to depression of tissue reactivity but to a depression of antibody production In one study, Cohen and Moses (47) induced passive sensitization of rabbits to horse serum and demonstrated some degree of modification by

very large amounts of cortisone of the arteritis resulting from horse serum injection

There are other allergic reactions which have not been mentioned They are much more difficult to study in the pure state because they involve multiple antigens Among these are the so-called bacterial allergic reactions, the reactions to tuberculin or to streptococcus nucleoprotein In humans, we have been unimpressed with the effect of corti- [illegible] reactions unless a [illegible] In one patient who had manifested glycosuria and [illegible] marked Cushing-like reaction, the skin became very thin and dry and probably would not have reacted to many stimuli. We have been unable to alter the tuberculin reaction in humans when milder doses of hormones are employed, yet such doses are sufficient to control the manifestations of an underlying disease such as rheumatoid arthritis or rheumatic fever

In guinea pigs, again, the inhibition seems to be a function of the dose of cortisone Independently, Stoerk (30), Long and Favour (48), Harris and Harris (49), and others, have reported complete suppression of the tuberculin reaction by using very large quantities of cortisone Our own experience is that moderate doses of cortisone do not appreciably inhibit the tuberculin reaction Five milligrams of cortisone a day administered to guinea pigs over a period of two months caused a slight diminution in the area of necrosis, but the area of erythema and induration seemed to be about the same. Also in connection with the so-called fixed tissue antibodies, it is proper again to mention the suppression of allergic encephalomyelitis by Kabat (36) in the monkey and Moyer (50) in the guinea pig. Kabat and his co-workers have very nicely demonstrated that the suppression may be due to a lack of reaction around the site of the injection of the vaccine, brain emulsion, and adjuvant so that, in effect, these animals act as though they never received the vaccine. It is a mistake to assume that the animals received the vaccine, had the expected reaction around the site of injection, and that the subsequent inflammation in the brain was then blocked by hormone at that later step of integration

That more or less concludes the discussion of the effect of adrenal hormones on hypersensitivity, but there are many reports in the literature which are worth mentioning. There is the problem of the role of connective tissue in antibody production, and the first question that suggests itself is what happens to antigen when it is injected? Dr. Coons and his co-workers have perhaps the most enlightening photographs on this subject. By tracing antigen in tissues, using a fluorescent-stained antibody to locate the antigen, they find that connective tissue does participate in the localization of antigen (51). There will certainly be fur-

ther studies of great interest on that score from his laboratory. In speculating as to what happens to antigen, it is hard to conceive of material going into a cell without causing a reaction, and there is some evidence that connective tissue cells may become sensitized, although the actual production of antibody in fibroblasts has not been demonstrated satisfactorily

Porter: What kind of cells take up the antigen, macrophages or fibroblasts?

Fischel Certainly macrophages, and the whole reticuloendothelial system, so-called. But even in connective tissue in coarse bundles, in fibroblasts, there seems to be some localization of antigen I imagine that it might depend on the degree of reactivity of that tissue, for instance, in old scar tissue, fibroblasts may not take up the antigen. That is a guess on my part

Porter: Does Dr Coons tag a protein with fluorescein dye?

Lillie: Fluorescein; isocyanate is used to conjugate

Fischel. With the antibody.

Lillie Protein antibody

Fischel: But I believe capsular polysaccharide of influenza was used Other people have traced bacteria or dye-labelled antigens into cell, but then they break up and it cannot be certain that they are traced further

Dempsey: Dr. Coons has used a number of different antigens, pneumococcus polysaccharide, I believe, and also some of the protein antigens

Fischel: Crystalline egg albumin and human γ-globulin

Porter. Is it inferred, then, that the antigen has to be taken up in order for antibody production to be stimulated?

Fischel. No, no! He injected antigen He actually used his antibody as a stain He washed the tissues from control animals to see how much of this labelled antibody would stick to cells nonspecifically and then to sensitized tissue By using appropriate light methods, he picked up the fluorescein dye which was fixed to the antibody, which in turn was fixed to the antigen It is much like what we were discussing previously about fixation of a heavy metal to a dye, which in turn might be picked up by both electron microscopy and classical microscopy

Lillie: Did he use ultraviolet?

Fischel: The antibody was conjugated with fluorescein

Porter: But he found that the antigen was inside the cells?

Dempsey: Particularly in the lymphocytes and, to some degree, in the macrophages

Fischel: I don't recall the lymphocytes.

very large amounts of cortisone of the arteritis resulting from horse serum injection

There are other allergic reactions which have not been mentioned They are much more difficult to study in the pure state because they involve multiple antigens. Among these are the so-called bacterial allergic reactions, the reactions to tuberculin or to streptococcus nucleoprotein In humans, we have been unimpressed with the effect of cortisone on tuberculin and streptococcus nucleoprotein reactions unless a very marked degree of hyperadrenalism is induced by cortisone. In one patient who had manifested glycosuria and dehydration and marked Cushing-like reaction, the skin became very thin and dry and probably would not have reacted to many stimuli. We have been unable to alter the tuberculin reaction in humans when milder doses of hormones are employed, yet such doses are sufficient to control the manifestations of an underlying disease such as rheumatoid arthritis or rheumatic fever.

In guinea pigs, again, the inhibition seems to be a function of the dose of cortisone Independently, Stoerk (30), Long and Favour (48), Harris and Harris (49), and others, have reported complete suppression of the tuberculin reaction by using very large quantities of cortisone Our own experience is that moderate doses of cortisone do not appreciably inhibit the tuberculin reaction Five milligrams of cortisone a day administered to guinea pigs over a period of two months caused a slight diminution in the area of necrosis, but the area of erythema and induration seemed to be about the same. Also in connection with the so-called fixed tissue antibodies, it is proper again to mention the suppression of allergic encephalomyelitis by Kabat (36) in the monkey and Moyer (50) in the guinea pig. Kabat and his co-workers have very nicely demonstrated that the suppression may be due to a lack of reaction around the site of the injection of the vaccine, brain emulsion, and adjuvant so that, in effect, these animals act as though they never received the vaccine It is a mistake to assume that the animals received the vaccine, had the expected reaction around the site of injection, and that the subsequent inflammation in the brain was then blocked by hormone at that later step of integration

That more or less concludes the discussion of the effect of adrenal hormones on hypersensitivity, but there are many reports in the literature which are worth mentioning There is the problem of the role of connective tissue in antibody production, and the first question that suggests itself is what happens to antigen when it is injected? Dr. Coons and his co-workers have perhaps the most enlightening photographs on this subject By tracing antigen in tissues, using a fluorescent-stained antibody to locate the antigen, they find that connective tissue does participate in the localization of antigen (51). There will certainly be fur-

ther studies of great interest on that score from his laboratory. In speculating as to what happens to antigen, it is hard to conceive of material going into a cell without causing a reaction, and there is some evidence that connective tissue cells may become sensitized, although the actual production of antibody in fibroblasts has not been demonstrated satisfactorily.

Porter: What kind of cells take up the antigen, macrophages or fibroblasts?

Fischel: Certainly macrophages, and the whole reticuloendothelial system, so-called But even in connective tissue in coarse bundles, in fibroblasts, there seems to be some localization of antigen I imagine that it might depend on the degree of reactivity of that tissue; for instance, in old scar tissue, fibroblasts may not take up the antigen That is a guess on my part.

Porter: Does Dr Coons tag a protein with fluorescein dye?

Lillie: Fluorescein; isocyanate is used to conjugate

Fischel With the antibody

Lillie Protein antibody

Fischel. But I believe capsular polysaccharide of influenza was used Other people have traced bacteria or dye-labelled antigens into cell, but then they break up and it cannot be certain that they are traced further.

Dempsey: Dr. Coons has used a number of different antigens, pneumococcus polysaccharide, I believe, and also some of the protein antigens

Fischel: Crystalline egg albumin and human γ-globulin

Porter: Is it inferred, then, that the antigen has to be taken up in order for antibody production to be stimulated?

Fischel No, no! He injected antigen He actually used his antibody as a stain He washed the tissues from control animals to see how much of this labelled antibody would stick to cells nonspecifically and then to sensitized tissue By using appropriate light methods, he picked up the fluorescein dye which was fixed to the antibody, which in turn was fixed to the antigen It is much like what we were discussing previously about fixation of a heavy metal to a dye, which in turn might be picked up by both electron microscopy and classical microscopy.

Lillie: Did he use ultraviolet?

Fischel: The antibody was conjugated with fluorescein

Porter: But he found that the antigen was inside the cells?

Dempsey: Particularly in the lymphocytes and, to some degree, in the macrophages.

Fischel: I don't recall the lymphocytes

Dempsey: Oh, I do, definitely. I agonized over their identification Very curiously, in this connection of relating hormones, particularly those of the adrenal cortex, to antibody formation, he also found an intense reaction in the cells of the adrenal cortex.

Fischel: Do you think that might be a factor due to the vascularity of those organs?

Dempsey: I haven't any idea what it is due to, but it is a striking phenomenon.

[illegible]

Dempsey: The interesting thing about the lymphocytes was that this traces the antigen into the cytoplasm.

Porter: How can you tell whether the fluorescein is on the inside or the surface of the lymphocyte?

Dempsey: By the usual microscopic methods: by depth of focus, by studying the difference in appearance of cells between those that have been cut across and those that are whole, those that present a whole surface and those that present a broken surface. Also, incidentally, by the fact that it appears as a granular material, scattered indiscriminately inside the cell cytoplasm, and the cell membrane can be distinguished and is blank The optical section of the cell, the cell membrane, does not show a ring.

Porter: The antibody complex might have been taken up after it was established.

Dempsey: No, not by this technique. Antigen is injected into the animal; the animal is killed and frozen sections are prepared and attached to a glass slide The antigen-antibody reaction then takes place in the section as it is dipped into the stain The cell is quite dead by that time

Lillie Although it is strictly a freezing technique, there is no chemical fixation interposed?

Dempsey: No

Holbrook: The antigen is still located by inference?

Dempsey: By its specific reaction with the tagged antibody.

Travell: There is an inference, though.

Fischel: Other antigens do not specifically react.

Dempsey: That is right; other antibodies also.

Fischel: And the tissue does not take up as much in the particular site

Dempsey: If the specific antibody is used, no other antigen will work with this particular setup, that is, if it is appropriately controlled It is not a matter of staining with inadvertent fluorescein

Robb-Smith: But it differs from the classic experiment in that, after all, it isn't the animal's own antibody which is being localized but the tagged antibody you marked which you see in the sections

Fischel: All they report is the tracing of antigens No comment was made about the possibility that antibody is necessarily produced every-

in the test tube.

Dempsey: That's right

Fischel: The classical literature on antibody production very nicely points, I think, to the reticuloendothelial system, to macrophages as contributing a great portion of antibody, the plasma cells being included in that category To localize to the cellular level of fibroblasts, with which this Conference may be more concerned, there are several studies which indicate that fibroblasts in tissue culture maintain some degree of sensitivity to specific antigens Therefore, the inference is that they probably contain antibody. Whether they manufacture it or take it out of the circulation and incorporate it is something to be speculated on

In 1928, Rich and Lewis (52, 53) published studies on the reactivity of fibroblasts to tuberculin when the tissue was taken from a tuberculin-sensitive animal. As you know, tuberculin does not appear to give rise to a bona fide antibody in the serum It seems to be a cellular antibody that can be transmitted Chase (54) has transmitted tuberculin sensitivity with macrophages, and the tissue culture work by Rich and Lewis, Aronson (55), Moen and Swift (56), and others, indicates that there is a definite sensitivity of fibroblasts to bacterial antigens Moen and Swift also worked with the hemolytic streptococcic sensitivity in tissue culture. I have no way of evaluating tissue culture work Dr Porter, can you tell me what you think?

Porter: I am sure it is all right.

Fischel: At the present time, there has been a progress report and work is still going on in the laboratory of Drs Holden, Seegal, and

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their results indicate, at least in guinea pig tissues, that there is augmentation of the toxicity when cortisone and P P D are given together in sublethal doses This may, perhaps, have been anticipated from the fact that cortisone does seem to inhibit granulation tissue and fibroblasts *in vivo*, as Dr. Ragan and his group have shown (58) Hence, a sub-

lethal dose of cortisone in tissue culture, added to a sublethal dose of P P D may possibly result in synergistic inhibition That is speculation, but Dr Holden's group has an extensive study in progress which seems to indicate it may be true Curiously enough, they find that rabbit tissue does not appear to be as sensitive as guinea pig tissue, perhaps in keeping with the observation that rabbits require much more tuberculin to give a tuberculin reaction in the skin than do guinea pigs.

Robb-Smith: What is the biochemical lesion in the cell that occurs when there is an antibody-antigen change? We know all about the gross morphology but why these changes?

Fischel· We naively approached that problem in just one short experiment last year before we were sidetracked, and we found no change in respiration, no difference in oxygen uptake of cells exposed to tuberculin or to egg albumin when the tissues were from guinea pigs who were sensitive to both But that is very crude because oxygen uptake, after all, is an end result

There has been a report on inhibition of cathepsin by antigen-antibody reaction of the tuberculin type (59). That should be a fruitful point of departure for research to discover the initial lesion before there is cell death and all the secondary effects. What is the initial lesion biochemically which is caused by intracellular antigen-antibody reaction?

Ragan: Isn't it well known that, before cell death occurs, the cells stop migrating?

Fischel· The migration in tissue culture is inhibited and the cells are shrunken, with resulting bizarre shapes, etc. That is the morphologic picture I think the question is fundamentally one on a biochemical level

Meyer· Has it been shown that histamine is produced or liberated intracellularly in the cellular type of hypersensitivity?

Fischel: Histamine plays a role in the type of reaction such as is caused by a circulating antibody-antigen, but I do not know whether it has ever been demonstrated, say, for the tuberculin reaction Although antihistaminics do not seem to inhibit tuberculin reactions, that is no criterion

Robb-Smith. There will be many nonimmunological reactions, won't there, with histamine release? That, in a way, is the late stage of what has happened to the cell. It is common to a great many things

Fischel. Why it is that the histamine came about in the first place?

Robb-Smith Yes; what do antigen and antibody do when they are in the cell or on a cell

Fischel: From the point of view of physics, what happens to a semipermeable membrane when it is stopped up with a lot of precipitate,

if you will? But that doesn't explain the nonprecipitable antigen-antibody reaction.

Porter: You are saying it is inside, though, not on the cell surface

Fischel: We don't know.

Robb-Smith: It could be either The question is what really happens

Fischel: For what it is worth, the migrating cells have sensitivity, as evidenced by their failure to migrate when the antigen is dropped on them Whether that reaction occurs on the membrane or in the cell is completely unknown

Robb-Smith: But it is a problem that should not be too difficult to attack.

Porter: It is a nice problem for electron microscopy, Dr Wyckoff

Dempsey: Is anything known about the effect of hormones upon the histamine released? Is there any tie-in?

Fischel: From our small study and those of other people on the induction of anaphylaxis at threshold levels in guinea pigs, which is pretty much related to release and effect of histamine on the bronchioles, there is indirect evidence that there is no inhibition of either histamine release or the reactivity of tissues to histamine

Holbrook Quite the reverse. Rose (60) has shown, at least so far as the urinary output is concerned, that on the administration of ACTH and cortisone, the urinary histamine frequently increases greatly

Dempsey Do you mean there is much more histamine liberated which spills out?

Holbrook: We do not know where it originates, but the urine may contain 100, 200, or 300 *per cent* as much histamine We have not confirmed that as yet, but that is Rose's report

Dempsey So cortisone must induce an increased formation of, or breakdown of, a precursor of histamine, and thus it must be released somehow.

Holbrook. Somehow, somewhere, the output of histamine is increased in the urine We face the same problem with his findings regarding histamine as we do with the amino acids studied by us We do not know whether it is due to inhibition of synthesis or increased breakdown of tissue

Fischel: Or both

Dempsey: Does anybody know where the histamine is in the body normally, what cells have histamine or its precursor?

Fischel I think histamine might be found in almost any cell that is damaged.

Dempsey: There is no evidence, then, implicating one cell type anywhere in the body as being a major source of histamine?

Fischel: Do you know that, Dr Meyer?

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Fischel: I think histamine might be found in almost any cell that is damaged.

Dempsey: There is no evidence, then, implicating one cell type anywhere in the body as being a major source of histamine?

Fischel: Do you know that, Dr Meyer?

Meyer: No, I don't. If I remember correctly, the highest concentration of the histidine decarboxylase has been found in liver and kidney. The question is whether in cells there is bound histamine or other amines of similar pharmacologic properties or only histidine which becomes decarboxylated, and whether bound histidine could be decarboxylated when present as an end group.

Holbrook: That would be a very attractive hypothesis and we considered it quite early in our studies. We hoped that there might be a reciprocal relationship between the urinary histidine and histamine. We have not, as yet, studied the urinary histamine, but, about the time we conceived the idea, Rose came out with his work showing that the histamine also was markedly increased with the administration of ACTH, so that was a little discouraging from the standpoint of finding the source of the histamine.

Dempsey: I might mention some unpublished work done by Dr Helen Graham of the Department of Pharmacology at Washington University. She has chased down the blood histamine and has shown that the basophils of the blood have many thousand times the concentration of histamine found in other of the blood cellular elements or plasma. Indeed, if I remember correctly, about three-quarters of the blood histamine is located in the basophils, which, as you know, occur in very small numbers. In the tissues, there is a close relative to the blood basophil, the mast cell, which furnishes a nice speculative source for the tissue histamine. I believe it also has been shown that mast cells in the tissues show morphologic changes upon exposure to cortisone. The human basophil may be somewhat different from that of the rodent, but the cell type is there in the human.

Meyer: Is this pharmacologically demonstrable histamine?

Dempsey: I am sorry to say that I do not know the technique by which she identified it. I think it is microchemical.

Holbrook: We have been rather unhappy about the methods for histamine.

Dempsey: Since this work originated in Dr. Lowry's department, I am quite sure that the method would be a chemical one.

Holbrook: Did I understand you to say, Dr. Robb-Smith, that it should not be too difficult to tackle the problem of what happens in the cell?

Robb-Smith: That was my suggestion, but I spoke as a morphologist and not as a cytochemist. However, it does seem to me that there are methods of approach to a knowledge of enzyme systems in cells, and that it is the sort of thing which, using the Linderstrom-Lang types of technique, one should be able to approach. Whether an answer is found is another matter. I do feel that this new line that has come up

with regard to the known relationship of heparinemia and the changes in the mast cells is quite an intriguing side point to it.

Holbrook: Yes, so do I.

Meyer: If the cathepsin is decreased, just the opposite effect might be expected, since histidine might have to be liberated before it can be decarboxylated

Robb-Smith: It is an extraordinary thing that everybody, as far as I know, seems to have fought shy of this They just happily talk about toxic agents and cells feeling poorly and so forth, and get away with it

Fischel: It is hard to know what dies first when you fry the cell

Robb-Smith: Yes, but, after all, the biochemists don't allow them to be fried before they start to look at them

Fischel: Sometimes they do

Dempsey: It is a drastic method

Fischel: For what they are worth, there are several studies on *in vitro* Schultz-Dale reactions to histamine or to antigen-antibody reactions They contain some discrepancies but I am not familiar enough with the Schultz-Dale reactions to appreciate why or where the discrepancies might be On the whole, there seems to be a preponderance of opinion that cortisone does not inhibit the contraction of the uterine horns caused either by antigen-antibody reaction or by histamine in the water bath Dr. Richard Nelson (61) has reported a rather interesting variant, namely, that the sensitivity of the uterine horn is not affected by cortisone but that the gut of a guinea pig, which also contracts due to histamine or antigen-antibody reaction, is protected from the effect of the stimulus by cortisone Whether that has to do with the cells that are present in intestinal tissue as opposed to uterine horn tissue, I do not know, but it may be in line with what has been hypothesized here about mast cells or other cells

Holbrook: Have you any clear-cut idea as to a basic difference in the antigen antibody reaction which will produce what we choose to call immunity and another which will produce anaphylaxis and death?

Fischel: Much wrangling has gone on in the literature, particularly in the field of tuberculosis, about factors related to allergy and those related to immunity The discussions could be simply clarified, perhaps too simply, by approaching them from the point of view of immunochemistry; that is, certain antibodies are immunizing, such as, perhaps, the anti-M substance of the streptococcus, because the streptococcus happens to be vulnerable in its M substance Other antibodies to streptococcus, such as the antibody to the group specific C substance, are not immunizing but will cause an inflammatory reaction I think much of the old argument is based on illogical reasoning, on attempts to draw a distinct difference between two processes that are chemically similar but

teleologically different in that one reaction benefits humans, another does not, and indeed a third may perform both functions to varying degrees, partly immunizing but with an attendant inflammatory allergic reaction

Holbrook: I am fully aware of that, but is there a difference in the basic antigen-antibody reaction, in general, in these two groups of conditions? I am not trying to get into immunology and allergy, which is a problem we will never solve.

Fischel. As far as we can generalize, there is no fundamental difference

Holbrook: That is my opinion.

Fischel: In hay fever, it is very interesting that there is a thermolabile antibody which is apparently responsible for the sensitivity reaction, but Loveless has demonstrated that, after immunization with pollen extract, there is a thermostable antibody which effectively blocks the allergic reaction There we are dealing, perhaps, with a simpler system of antigens than in tuberculosis, but still not a single substance.

To avoid leaving the impression that it is thought there is great significance to this antibody depression by cortisone, it should be stressed that the extent of depression is really small and that it is probably not the factor which operates appreciably in clinical allergies That can be seen very easily in a patient with asthma or hay fever whose symptoms are controlled by cortisone and yet who has enough reagin or antibody present to give a very good skin reaction. It brings up the problem, again, of tissue reactivity and whether the inflamed tissues of a hay-fever-sensitive person may be soothed, if you will, by cortisone or inhibited from reacting to pollen But the normal tissue of the skin, when exposed to ragweed extract, will, either because of the extent of stimulus in a pinpoint area, the localization of a severe stimulus, or other reasons, react just as it did before cortisone was given That observation has been reported by many investigators Therefore, the clinical efficacy of cortisone probably has little relationship to antibody inhibition, except in such instances as reported by Damashek (62), where hemolytic reactions, hemoagglutination reactions of the autohemolysin type, were inhibited by cortisone, with a coincident disappearance of the abnormal antibodies

Holbrook In the problem of research in connective tissues, what people are thinking may be equally as important as what people are doing. We might ask ourselves several questions· Are there promising research problems which can be attached by our present techniques but are not being studied? Are there gaps in our knowledge due to lack of suitable techniques which need developing? Why is there so very little cooperation between the histologist, the chemist, and the physiologist?

Probably each one of us has thought of problems that need doing but which may not be entirely within our own field This is a good time for speculation, and I should like to have this group propose some new fields of investigation and perhaps stimulate the development of better techniques needed for the study Dr Robb-Smith made one most interesting suggestion I should like to hear some more comment on what we need to do to get along faster in our understanding of connective tissues Dr Ragan, what problem, one that you do not expect to undertake yourself, would you most like to see investigated?

Ragan: Our particular interest is in repair and inflammatory processes, through which we hope to understand something about turnover of tissues If some physical measurements could be applied to these particular processes, it might be most useful For instance, all the measurements of pH in tissue are open to criticism Some physicist might be able to develop a simple technique When an animal is killed, the pH drops off immediately, and the findings of electrodes are extremely questionable

Holbrook: The possible combination of some of the selective metal staining with ultramicroscopy as a solution to that has been mentioned

Lillie: That would require a lot of synthesis

Dempsey: Intellectual or chemical?

Lillie Both I should like to see very considerable efforts made toward finding out the meaning of some of the empirical staining methods which I described If a point could be reached where more is known about what they mean biochemically, they would be of greater value

Holbrook Amen

Ragan: Can they be applied to a test tube, for instance?

Lillie Where an already colored product stays in solution and an amino acid also stays in solution, how is it going to be determined whether they are in any combination?

Dempsey It is possible in some cases because there are a great many dye reactions in which the color acid has an absorption spectrum at one peak and a salt of the compound has a shifted absorption peak Therefore, the amount of the dye in its salt form and in its free acid form can be measured in solution and it can be known how much is combined A number of colorimetric processes of that kind can be used, and, indeed, I have been doing some work of this type in tissue sections in an attempt to find out whether the dye is reacting with the tissue through a chemical salt bond or whether it is held by some other force in the tissue There is considerable territory in that direction which could be explored

Lillie: One could explore what happens when mixtures of the more

common amino acids and collagen are made with the more selective of the triphenyl-methane acid anilin dyes.

Ragan: I was talking to Dr. Meyer about this a long time ago Would not an insoluble dye give a color which could be detected in a test tube?

Lillie: Yes, but what we want to know is what part of the collagen is giving the color.

Ragan Yes, but, to my knowledge, nobody has even carried out such a technique on collagen, have they, Dr. Meyer?

Meyer: There has been work of that kind done, but not with soluble collagen It would depend on whether it is done with molecules which are freely distributed or fixed in a definite position; each certainly will have different effects.

Lillie: We have already done it with gelatin.

Meyer. I am sure the effects you get with gelatin have very little to do with what you see in the staining technique.

Lillie. We know that the acid dyes will collect on the gelatin

Meyer We could go all over the problem again which was presented yesterday in Dr Wyckoff's paper and the discussion; namely, what is the meaning of the banded structures If one has a detailed molecular knowledge, it can be applied in attempts to interpret the data from the electron microscope; the attempts would have to be preceded by a study of the rationale and an explanation of the x-ray diffraction pattern, which is sorely lacking You see, if you figure out the cross section, no matter how, of this peptide chain and see that it is 200 Å wide, the smallest fibril which you can see in the electron microscope is in the collagen fiber

Wyckoff That is almost the smallest one we see, but perhaps not the smallest

Meyer: It is still more important, naturally, to know that there is none smaller than 200 Angstrom Units. If you figure from the valence bond of the peptide bond and the kinking which you would get from the proline or from the amino acid, the peptides, and the whole backbone, I don't know what the figure would be, but you wouldn't arrive at more than a few Angstrom Units.

Robb-Smith: A diagram might be helpful (*Figure 50*)

Meyer: The immediate consequence is that there must be, if they are arranged in parallel, a cross section of 200, or there must be quite a large number of amino acids which could cross-sect. How are they arranged in relation to each other? This whole problem of specific reactions of stains is so complex because there is not only salt formation but winding by van der Waal's forces and other formations. One of the most important things, which I think we never think about, is that if

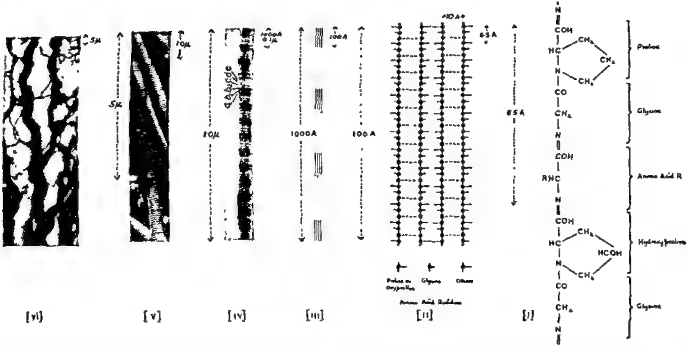

FIGURE 30 Diagram illustrating the size relationships between collagen fibers as seen under the light and electron microscopes and schematic representations of collagen grid and molecular pattern of the collagen chains

i Schema for the molecular pattern of collagen chains x 100 000 000
ii Schema for the collagen grid x 10 000 000 (modified from Astbury, W T *Journal Int Soc Leath Chem* 24, 69 (1940)
iii Tenfold reduction of the collagen grid x 1,000,000
iv *Electron micrograph* of collagen fiber stained with phosphotungstic acid x 100,000 Reprinted by permission from Gross, *J*, and *Schmitt*, F O Structure of human skin collagen as studied with the electron microscope *J Exper Med* 89, 555 (1948)
v Electron micrograph of collagen fibers shadowed with chromium x 14 000
vi Photomicrograph of collagen and reticulin, silver impregnation x 1,000

we apply a dye, the dye very often must replace something else If basic dyes are combined, for example, which give metachromasia *in vitro*, they replace something else. What is that something else which they replace? We may reach a completely erroneous interpretation by not figuring competition between compounds in the tissue This is purely speculative. I have no experience in histology except what I learned in medical school, and in my time that wasn't much

Fremont-Smith: I should like to guess that you are right, that the last thing we are going to understand is how any of the standard stains work They are extremely complex and perhaps new stains will be developed which will be better understood before the older ones are

Lillie. We can conceive of a great many of the stains being rather simple sulfonic acid unions with amino groups

Meyer: But what neutralized the amino group? I assume you take a basic acidic group, what was it before you applied the dye? Was it a sodium ion? Or a protein?

Dempsey. That has been worked out in a number of basic staining reactions in which the pH drops as the dye is taken up Thus, hydrogen ion is given off so the dye replaces hydrogen ions.

Meyer: If you had the collagen as a basic protein, with, I believe, an isoelectric point of around 7, this basic protein is in close approximation, let us say, in the tissue, in tendon, with other proteins and with polysaccharides What is the arrangement? I am sure that a shift in hydrogen ion concentration would not be found because replacing, let us say, an albumin molecule by toluidine blue does not produce a shift in pH

Robb-Smith. I entirely agree with Dr Fremont-Smith. Some preparations where we got what we call hyalin, which is a funny sort of collagen, apparently stained perfectly well with the trichrome stain in specimens treated with "collagenase." But there isn't any collagen there at all That is the sort of catch you can get The sections look all right but they have been treated with an enzyme and the particular substance has been broken down and is no longer there.

Meyer: A much more beautiful example is the nucleus It is assumed that the nucleus is stained if basic dyes are used

Lillie: Yes

Meyer. This nucleus is desoxyribonucleic acid By actual measurement, the amount of the concentration of desoxyribonucleic acid is about 10 or 12 per cent The rest is protein The protein is not stained at all The histologist concludes that that nucleus is composed of desoxyribonucleic acid

Dempsey: Oh, no, quite the contrary, Dr. Meyer. The histologist stains the nucleus with the basic dye, and he concludes that the nucleic

acid component of the nucleus stains with this dye. Then he carries on another experiment in which he stains the nucleus with an acid dye and concludes that this is staining the protein Then he may destroy the nucleic acid by digesting the section in desoxyribonuclease and find that this has destroyed the basophilia of the nucleus but the acidophilia is unimpaired, which fortifies his conclusion that the protein is still there No histologist is so naive as to believe that any structure he sees is a pure compound.

Meyer: No, no, it may not be a pure compound, but what happens to the protein, which I assume neutralizes the hydrogen ions of the phosphate groups, the free phosphate groups, in the nucleic acid?

Dempsey What happens to it?

Meyer: Yes; what happens to the protein if you apply the stain? Does it go out of the cell?

Dempsey. No, it remains precipitated It is all in the solid phase to begin with, and it remains in the solid phase The mechanism one ordinarily thinks of is that the bond between the nucleic acid and the basic protein has been forced in much the same way that it is forced with a high concentration of salt solution or a change of pH of the solution In other words, the nucleic acid can be separated from the nucleoprotein by a number of chemical methods which are quite analogous to those used in staining

Lillie: Or those used in converting phosphate to carbonate

Wyckoff: I should like to say something I am afraid that the two electron microscopists here may have rather failed to make clear how much there is yet to be learned about what may be called the ultramorphology of connective tissue Our discussion has been, I think, a demonstration of how very, very little we now know about the fine structure of the various connective tissue elements in the animal I hope you are convinced that the problem can be experimentally attacked, but all our evidence shows, beyond everything else, its own relative poverty

We have made experiments on tendon, on the connective tissue elements developed in tissue culture which Dr Porter has done, and on the products of fibroblast activity in one or two very special localities But we know almost nothing about the connective tissue distributed through the organs of the body This is, by and large, the most important part of the job and one which is not up to the expert in electron microscopy to attempt, for, in this area, we are complete amateurs I am not talking for Dr Porter now because, after all, he is a cytologist, and rather an exception The rest of us, as I say, are and always will be amateurs, and the work has got to be done by you professionals, not by us

Fremont-Smith: What kind of new cooperation is needed to do that?

Wyckoff. I think what are needed are a few histologists who have become experts in the study of fine structure. I don't think the problems will ever be solved simply by joint cooperation between electron microscopists and conventional histologists Progress must come from histologists whose primary problem is the structure of tissues and who, to meet this problem, have become competent themselves in handling this new tool for approaching it. There is too great a tendency to have a division of labor, with a histologist providing one technique and the electron microscopist another. Genuine results come only when the man who is attacking the fundamental problem has become expert with the necessary tools.

Dempsey: There are very few electron microscopes in the charge of people who are professional histologists.

Holbrook: Why is that?

Wyckoff: They are in the charge of technicians. That is a mistake which has been made throughout in the use of the electron microscope, and the reason why the microscope has accomplished so little of what it could have in the ten years of its existence. Too often the men with the problems have delegated the observation to somebody else, but somebody else's eyes cannot be used It is generally true in any survey of nature that the man who knows the problem must make the observations if they are to be significant; this is especially true of electron microscopy because many fields must be scanned at high magnification in order to cover a representative sample of what is there, and nobody can tell someone else how to look for representative material. No more than a hundredth part of what is there can be photographed, and only the man who knows the material recognizes something significant when he sees it.

Holbrook. I think we all might bear that in mind.

Meyer: May I say that I also have a pet wish. It is about the cell, the fibroblast or the connective tissue cell; that is, whether there is any possibility, histologically or by any other method, to differentiate between cells; what, in detail, are the compounds of the connective tissue elements which they obviously or presumably produce. Another thing, for example, would be to explain such observations as that the skin in the baboon will respond to estrogen on certain sites and not on others In the cock's comb, tissue cells respond to androgen, and it is not known whether the other cells respond to it. Is there any chance of getting at a histologic basis for biochemical differentiation?

Robb-Smith: Couldn't it possibly be done another way, that is, following from Dr. Porter's work, by taking tissue cultures of cells that are producing fibers and seeing how they get on in various types of media? It might be discovered what substrates they require and, pos-

sibly, the various types of inhibitors and so forth Then there would be a controlled environment for their production Fibers produced in a controlled environment could be studied morphologically in the electron microscope to see when the cells made bad fibers, none at all, or good ones. I think that sort of approach would be helpful because I feel that in animal tissue we are up against the difficulty of these things depending on the substrates which are in the environment and the enzymes the cells are forming. In other words, according to their substrate, they build up different enzyme systems and form different substances, some of which are catabolic products, although they may be very important ones from the point of view of what we call bacterial toxins or cellular toxins, and others of them may be substances which are determined in normal development

Meyer: What comes first in the sex skin; the environmental changes? Why does the environment change only in that locality and not in some other site?

Robb-Smith. Because presumably that is the area in which there is already a particular type of substrate One doesn't know what happens to the cell, if transplant experiments are done I think you can transplant from tissue to tissue and it doesn't work

Dempsey: There is a beautiful experiment in which you can find a location to study this phenomenon biochemically, if you like, Dr Meyer. The tadpole has no legs. Give it a little thyroxin and immediately, in certain localized areas of its body, it begins to sprout legs The skin covering these areas is transformed very rapidly and very dramatically upon exposure to the thyroxin If a section is made in that area, an absolute cell boundary can be delimited, on one side of which the tissue is responding and on the other side of which the tissue is not responding

Wyckoff. Do the legs develop in the right places?

Dempsey: Oh, yes.

Wyckoff: And only in the right places?

Dempsey: That's right, only in the right places There is a series of papers in the French literature in which a suggestion is made that the parasympathetic ganglia supplying these cells, which metamorphose or which are susceptible to endocrine stimulation, are different from those parasympathetic cells stimulating other regions

Ragan: Is the thyroxin simply speeding up maturation?

Dempsey: Yes Maturation never occurs without the thyroid The tadpoles will invariably remain in the larval form

Ragan: These are thyroidectomized tadpoles?

Dempsey: Yes The thyroidectomized tadpoles or tadpoles fed thiouracil will never metamorphose; they will always have a tail and

never a leg. Give them thyroxin and they will develop legs

Ragan: But isn't the problem why does a tadpole develop legs there anyway?

Dempsey: It is exposure to an endocrine substance which precipitates the thing that it is capable of doing.

Lillie. That is a question of mechanism.

Meyer. Yes, but in specific areas, namely, sex skin and comb, all you do, it seems to me, is to make conjugative changes. The whole skin fibroblast presumably produces hyaluronic acid, for example. They produce much more under the stimulus in that localized area.

Dempsey: That same cell is probably producing hyaluronic acid and doing something that causes reticular fibers to form, either in itself or immediately adjacent to itself, and elastic fibers and goodness knows what else

Meyer Yes, but you only speed it up You do not create, as in the egg cell, where you really cause new formation of totally different proteins

Dempsey. Well, your statement that it is merely a quantitative change is a much more dogmatic one than I would want to make.

I should like to comment that Dr. Meyer has done quite a little in asking us to develop methods that will differentiate between the products of cells. For example, the egg cell, I believe, produces a rather astonishing number of products before it gets through. So he has really thrown the entire load of biochemistry and, indeed, the study of life onto the histologists, and I think we ought to get out from under somehow

Robb-Smith: That is why I threw it back to him

Lillie. We have to think about adapting some of the analytical methods to the peculiar conditions under which we work.

Meyer· Your objection to the egg cell, I think, is not completely valid

Dempsey· I mean that cells do more than one thing We cannot say that here is a cell and it has a specific function and now all we have to do is develop a method which will show us what the quantitative activity of that function of the cell is. The cell is living, the cell is manufacturing specific secretions, it is reproducing itself, it is elaborating new mitochondria and new Golgi apparatus and doing a great many things simultaneously It is a very complex problem, not a simple one

May I comment on what I think is really the dilemma confronting both histologists and biochemists? The biochemist has a long tradition of quantitative methods, of methods which allow him to make numerical measurements, and of results which he can handle mathematically The morphologist, on the contrary, has a long tradition of localizing

visible substances spatially in the body. His methods are not susceptible to mathematical treatment except in rare cases and so he is occupied in an almost qualitatively different type of study. Now, in between these two very different disciplines, there has grown up a subject which, for want of a better name, we call histochemistry, in which the morphologist is trying to learn how to apply the intellectual disciplines of the chemist to his problems and in which the chemist is trying to learn how to apply the morphologic disciplines We are moving in each other's direction but we have not yet reached each other's positions As an example, I can mention that Dr Lowry, in our school, has been concerned with microchemical methods for some time and we have had fun in calculating the minimum quantity of material that he can analyze by quantitatively respectable chemical methods When we do that, we discover that the minimum amount of tissue from which this material must be forthcoming comprises anywhere from a few cells to many, many hundreds of cells Consequently, quantitative methods are not yet applicable to problems of cytological organization They are too crude They do not yet have the sensitivity necessary to localize sharply in space the compounds that are being studied

Conversely, the morphologist, who isolates or who sees a granule or a fiber a tenth of a micron in diameter, is not even able to measure quantitatively how many of these fibers there are, let alone the chemical composition of the fibers. He has a method that will sharply pinpoint something in space but which will not give him a local concentration In between, there is this difficult gap which has not yet been filled That is where almost all of the trouble arises

The questions to which we should like to have the answers lie in this territory We should like to know, I think, what the quantitative concentration of an enzyme is in the living cell in which it is functioning Chemical analyses of the total amount present in a sample of 100 mg. of tissue will not answer that question because, after all, the enzyme may be restricted to one little ball in those 100 mg of tissues On the other hand, cytological studies will not answer it either because only by inference can we guess that the thing we see actually represents the enzyme Therefore, I think the answer to Dr Holbrook s question lies in striving to close the gap between chemistry and morphology This is exactly what we are trying to do, but we just don't know how to do it well enough

Fremont-Smith· There are many such gaps, and I think you are right that it is by one person learning to span two techniques with respect to a problem he cares about that we can eventually build this bridge

Wyckoff· That's right The emphasis has to be on problem, not on technique

Lillie: It is perhaps unfortunate that biochemists have approached many of their problems by first dissolving the thing that they want to analyze. In order to understand the solid state, you have to be careful not to dissolve it.

Robb-Smith: Following on Dr. Dempsey's observations with which I entirely agree, it would be quite profitable, I believe, to sit down and just see how much spatial biochemistry one could write; in other words, how much knowledge is there, say, of the muscle fiber? How much does one know?

Dempsey: Some things we could specify. It would be important to know what they were

Robb-Smith: We could do it not too badly in places. We would find other chapters that were just blank pages That would be a profitable exercise I am not suggesting that anybody around this table undertake it, but it would be a good frontier point to see just how far we have gotten at this stage

Fremont-Smith: And those blank pages would not be altogether blank in the other disciplines.

Robb-Smith: If we are talking as Dr Dempsey was, we don't bother with a discipline itself, really.

Fremont-Smith: You said spatial biochemistry. If we were limited to that, we would find many blank pages, but histochemistry and staining might fill in some of those pages.

Robb-Smith: Yes, that's right.

Holbrook: I think this is a good note on which to close.

Fischel: One small point, and that is, after morphology and chemistry, we still have a huge gap to fill in respect to physiology and function

Holbrook: Yes, that is correct. Thank you all very much for your attendance and your attention and your courtesy.

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